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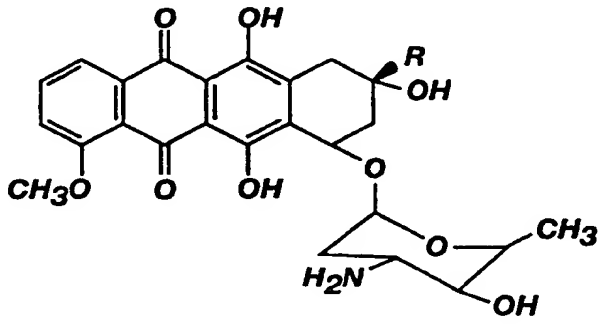

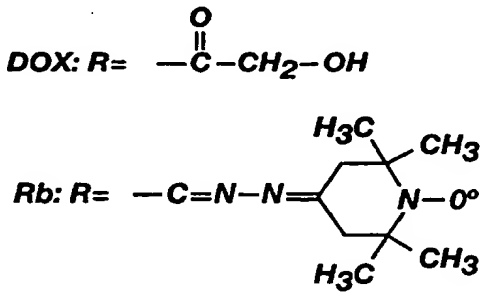

(57) Abstract

A method for administering a drug to a selected site in a patient includes the steps of (a) administering a composition including a micellar drug carrier having a hydrophobic core and an effective amount of the drug disposed in the hydrophobic core; and (b) applying ultrasonic energy to the selected site such that the drug is released from the hydrophobic core to the selected site. Preferably, the drug carrier is a triblock copolymer, such as a poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) block copolymer having a molecular weight of about 6500. The drug is preferably an antineoplastic agent such as doxorubicin.

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ACOUSTICALLY ACTIVATED LOCALIZED DRUG DELIVERY

Two Sided

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/059,774, filed September, 23, 1997.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under Grant No. RO1 HL-52216 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

This invention relates to drug delivery. More particularly, the invention relates to ultrasonically enhanced drug delivery using micellar drug carriers.

The efficacy of cancer chemotherapy is often limited by toxic side effects of the anticancer drugs. An ideal scenario would be to sequester the drug in a package that would have minimal interaction with healthy cells and would contain the drug until release. Then, at an appropriate time the drug would be released from the sequestering container at the tumor site. To achieve this goal, various long-circulating colloid drug delivery systems have been designed during the past three decades. A common structural motif of all these long-circulating systems, whether they be nanoparticles, liposomes, or micelles, is the presence of poly(ethylene oxide) (PEO) at their surfaces. The dynamic PEO chains prevent particle opsonization and render them "unrecognizable" by the reticuloendothelial system (RES) of cells (S.I. Jeon et al., 142 Colloid Interface Sci. 149-158 (1991)). This advantage has promoted extensive research to develop new techniques to coat particles with PEO, techniques ranging from physical adsorption to chemical conjugation.

From a technological perspective, polymeric micelles formed by hydrophobic-hydrophilic block copolymers, with the hydrophilic blocks comprised of PEO chains, are very attractive drug carriers. These micelles have a spherical, core-shell structure with the hydrophobic block forming the core of the micelle and

the hydrophilic block or blocks forming the shell. Block copolymer micelles have promising properties as drug carriers in terms of their size and architecture. Only a few known block copolymers, however, form micelles in aqueous solutions. Among them, AB-type block copolymers (e.g. poly(L-amino acid)-co-poly(ethylene oxide), M. Yokoyama et al., 51 Cancer Res. 3229-3236 (1991); A.V. Kabanov et al., 22 J. Controlled Rel. 141-158 (1992); G. Kwon et al., 9 Langmuir (1993); G.S. Kwon et al., 10 Pharma. Res. (970-974 (1993); G.S. Kwon et al., 6th Int'l Symp. On Recent Advantages in Drug Delivery Systems 175-176 (1993); G.S. Kwon & K. Kataoka, 16 Adv. Drug Delivery Rev. 295-309 (1995)) and ABA-type triblock copolymers (e.g. A.V. Kabanov et al., 22 J. Controlled Rel. 141-158 (1992); V.Y. Alakhov et al., First Int'l Symp. On Polymer Therapeutics 213 (Univ. London 1996); A.V. Kabanov et al., 28 Macromolecules 2303-2314 (1995); 113 J. Magn. Res. A 65-73 (1995); N. Rapoport & K. Caldwell, 3 Colloids & Surfaces B: Biointerfaces 217-228 (1994); N. Rapoport, Eleventh Int'l Symp. On Surfactants in Solution 183 (Jerusalem 1996)) deserve special attention. The PLURONIC family of ABA-type triblock copolymers has the structure PEO-PPO-PEO, where PPO is poly(propylene oxide). The hydrophobic central PPO block forms a micelle core, and the flanking PEO blocks form the shell or corona that protects micelles from recognition by the RES.

Several advantages of polymeric micellar drug delivery systems include: (1) long circulation time in the blood and stability in biological fluids; (2) appropriate size (10-30 nm) to escape renal excretion but to allow for extravasation at the tumor site; (3) simplicity in drug incorporation compared to covalent bonding of the drug to a polymeric carrier; and (4) drug delivery independent of drug character.

Some micellar systems are dynamically stable because their solid-like cores dissociate slowly at concentrations below their critical micelle concentration (CMC) (M. Yokoyama et al., 10 Pharma. Res. 895 -899 (1993); K. Kataoka et al., 24 J. Controlled Rel. 119-132 (1993); A. Halperin & S. Alexander, 22 Macromolecules 2403-2412 (1989)). Others are not stable and require additional stabilization that may be achieved, for instance by cross-linking the micelle core (A. Rolland et al., 44 J. Appl. Polym. Sci. 1195-1203 (1992)).

In a study of pharmacokinetics and distribution of doxorubicin in micelles formed by drug-polymer conjugates, the conjugate circulated in the form of micelles

much longer in blood than did free drug (M. Yokoyama, 17th Int'l Symp. On Recent Advantages in Drug Delivery Systems 99-102 (1995)). The uptake of the conjugated drug by various organs proceeded much slower than that of a free drug, and lower levels of conjugate were found in the heart, lung, and liver compared to much higher conjugate level in the tumor (M. Yokoyama, Advances in Polymeric Systems for Drug Delivery (1994)).

Cross-resistance to anti-cancer drugs in malignant cells is also a major problem for chemotherapy (M.S. Sanford & S. Melvin, 91 Proc. Nat'l Acad. Sci. USA 3497 (1994)). Despite an initial favorable response to chemotherapy, almost 50% of patients relapse, and the recurrence of the disease is often associated with clinical drug resistance (P. Maslak et al., 17 Cytometry 84 (1994)). The most common resistance mechanism is increased drug efflux due to amplification of the gene for P-glycoprotein (R.L. Juliano & V. Ling, 445 Biochim. Biophys. Acta 152 (1976); J.L. Biedler & H. Riehm, 30 Cancer Res. 1174 (1970); G. Bradley et al., 948 Biochim. Biophys. Acta 87 (1988)). P-glycoprotein (P-gp) is situated in plasma membranes and acts as an energy-dependent drug-efflux pump producing decreased drug accumulation within the cells.

Several attempts have been made to overcome resistance in cancer cells. Drugs such as verapamil have been shown to modulate P-gp activity by inhibiting the binding of some anti-neoplastic drugs to P-gp (T. Tsuruo et al., 42 Cancer Res. 4730 (1982)). Although a number of other agents have been shown to reverse the multiple drug resistance (MDR) phenotype (J.A. Moscow et al., Multi Drug Resistance, in Cancer Chemotherapy and Biological Response Modifiers 91 (H.M. Pinedo et al. eds. 1992)), their clinical applicability toward resistant tumors has been restricted due to their toxicities (U. Consoli et al., 88 Blood 633-644 (1996)).

Several other methods have been proposed to overcome drug resistance, based on bypassing the P-gp pump such as drug delivery in liposomes, combined delivery of drugs and surfactants, delivery in micelles, and delivery of polymer-drug conjugates.

Ultrasound has been used extensively for medical diagnostics and physical therapy. An advantage of ultrasound lies in the fact that it is non-invasive, and the energy can be controlled and focused easily, with the capability to penetrate deep

into the tissue. Several reports have demonstrated enhanced cytotoxic response when ultrasound and chemotherapeutic agents were combined (R. Jeffers, 98 J. Acoust. Soc. Am. 2380 (1995); V. Mislik et al., 25 Free Radical Res. 13-22 (1996); V. Mislik et al., 20 Free Radical Biology and Medicine 129-138 (1996); all of which are hereby incorporated by reference). The most prominent manifestation of this drug-ultrasound synergy was an increased drug uptake. There are also several hypotheses reported in the literature regarding the mechanism of ultrasonic enhanced activity of anthracycline drugs (A.H. Saad & G.M. Hahn, Heat Transfer in Bioengineering and Medicine (J.C. Chato et al. eds. 1987); A.H. Saad & G.M. Hahn, 49 Cancer Res. 5931-5934 (1989); A.J. Saad & G.M. Hahn, 18 Ultrasound Med. Biol. 715-723 (1992); R.J. Jeffers, Activation of Anti-cancer Drugs with Ultrasound, Ph.D. Dissertation, Univ. of Michigan (1995); P. Loverock et al., 63 Br. J. Radiol. 542-546 (1990); D.B. Tata et al., 3 Ultrasonics Sonochemistry 39-45 (1996); all of which are hereby incorporated by reference). These reports are mainly concerned with acoustic-induced hypersensitization of drug-sensitive lines.

To suppress side effects to normal tissue and to improve the efficiency towards the cancerous cells, targeting of these drugs using several types of drug carriers has been studied. The recent efforts towards designing such types of delivery systems have led to the development of delivery vehicles that are more stable in the blood system compared to previous carriers that were rapidly taken up by the reticuloendothelial system. Poly(ethylene oxide) (PEO) is a common structural component of these new drug carriers. It is a well known biomedical polymer, expresses low toxicity, and when present at surfaces and interfaces, it has the ability to suppress cellular and protein adsorption (G.S. Kwon et al., 2 Colloids Surfaces B: Biointerfaces 429-434 (1994)). Poly(oxyethylene-*b*-oxypropylene-*b*-oxyethylene) triblock copolymers represent non-toxic polymeric surfactants that have been used in a number of drug targeting applications (V.Y. Alakhov et al., 7 Bioconjugate Chem. 209-216 (1996); A.V. Kabanov et al., 22 J. Controlled Rel. 141-158 (1994)). These triblock polymers attract special attention due to their low toxicity and ability to solubilize biologically active lipophilic substances (I.R. Shmolka, 54 J. Am. Oil chem. Soc. 110-116 (1977); E.W. Merril, Poly(ethylene oxide) and Blood Contact, in Poly(Ethylene Glycol) Chemistry 199-220 (J.M. Harris

ed 1992)). The concept underlying these polymers is the principle that the structure formed with amphiphatic molecules will, in aqueous medium, present their hydrophilic (PEO) portion to the external aqueous media, while the hydrophobic parts (polypropylene oxide; hereinafter "PPO") will be oriented towards the internal part of the structure. The hydrophobic drug molecules would then partition inside the micelles. It has been suggested that these 15-35 nm diameter carriers can enter the cells by phagocytosis or endocytosis, and the drug can be delivered inside the cells by local delivery or by fusion with the membrane, thereby destabilizing it (R. Paradis et al., 5 Int. J. Oncol. 1305-1308 (1994)).

The structural transitions of one such triblock copolymer (PLURONIC P-105) has been reported (N. Rapoport & K. Caldwell, 3 Colloids and Surfaces B: Biointerfaces 217-228 (1994)). The transition was shown to proceed from unimers to loose hydrated aggregates to stable dense micelles with a hydrophobic core. The onset of multimolecular micelles was shown to correspond to a concentration of 1 wt% of PLURONIC P-105 and was completed at 10 wt%, with two populations of micelles co-existing at intermediate concentrations. The solubilization efficiency of PLURONIC for hydrophobic or amphiphilic molecules was found to increase dramatically upon formation of dense micelles.

In view of the foregoing, it will be appreciated that providing a method for delivering drugs that avoids or reduces the side effects and multiple drug resistance phenomenon associated with many chemotherapeutic agents would be a significant advancement in the art.

BRIEF SUMMARY OF THE INVENTION

It is an object of the present invention to provide a method for delivering chemotherapeutic agents that avoids or reduces side effects and multiple drug resistance associated therewith.

It is also an object of the invention to provide a drug delivery composition for treating cancer.

It is another object of the invention to provide a method for delivering hydrophobic therapeutic agents by encapsulation in micelles in conjunction with ultrasound.

These and other objects can be addressed by providing a method for delivery of a drug to a selected site in a patient comprising the steps of:

(a) administering to the patient a composition comprising a micellar drug carrier having a hydrophobic core and an effective amount of the drug disposed in the hydrophobic core; and

(b) applying ultrasonic energy to the selected site such that the drug is released from the hydrophobic core to the selected site.

A composition for delivery of a drug to a selected site in a patient comprises a micellar drug carrier having a hydrophobic core and an effective amount of the drug disposed in said hydrophobic core. Preferably, the micellar drug carrier is an ABA-triblock copolymer, more preferably a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer. The drug is preferably a hydrophobic drug or a drug having a hydrophobic center such that it can be sequestered in the hydrophobic core of the micellar carrier. Illustrative drugs include doxorubicin and ruboxyl.

A method for enhancing uptake of a drug by cells at a selected site in a patient comprises the steps of:

(a) administering to the patient a composition comprising a micellar drug carrier having a hydrophobic core and an effective amount of the drug disposed in the hydrophobic core; and

(b) applying ultrasonic energy to the selected site such that the drug is released from the hydrophobic core and taken up by the cells.

A method for reducing side effects in a patient from administration of a drug comprises the steps of:

(a) administering to the patient a composition comprising a micellar drug carrier having a hydrophobic core and an effective amount of the drug disposed in the hydrophobic core; and

(b) applying ultrasonic energy to the patient such that the drug is released from the hydrophobic core.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

FIG. 1 shows the concentration of solubilized DSTA as a function of PLURONIC P-105 concentration; EPR spectra before and after dense micelle formation are shown in the left inset and right inset, respectively.

FIG. 2 shows the structure of doxorubicin (DOX) and ruboxyl (Rb).

FIG. 3 shows Stern-Volmer plots for free doxorubicin (□), doxorubucin in 0.1 wt% PLURONIC P-105 (♦), doxorubicin in 1 wt% PLURONIC P-105 (▲), doxorubinin in 10 wt% PLURONIC P-105 (▼), and doxorubicin in 20 wt% PLURONIC P-15 (●), respectively, wherein the concentration of doxorubicin was 5 μg/ml; the inset plot shows K_{SV} versus concentration of PLURONIC P-105.

FIG. 4 shows growth curves of human HL-60 leukemia cells preincubated for 6 hours with 0% (□), 0.1 wt % (♦), 1 wt % (●), or 10 wt % (▲) PLURONIC P-105.

FIG. 5 shows growth curves of HL-60 cells treated for 6 hours with ultrasound and 0% (□), 0.1 wt % (♦), 1 wt % (●), or 10 wt % (▲) PLURONIC P-105.

FIG. 6 shows survival curves of HL-60 cells treated for selected times with PLURONIC P-105 and ultrasound; cell counts were taken at 72 hours post-treatment: (■) 1 hour incubation, (●) 1 hour sonication, (♦) 6 hour incubation, (▲) 6 hour sonication.

FIG. 7 shows the cytotoxicity of free doxorubicin (■), doxorubicin in micelles (♦), free doxorubicin with ultrasound (●), and doxorubicin in micelles with ultrasound (▲); the inset shows a plot of IC_{50} values versus the different treatments.

FIG. 8 shows drug accumulation in HL-60 cells following treatment with 5 μg/ml of doxorubicin (Free), 5 μg/ml doxorubicin with ultrasound (Free/US), 5 μg/ml doxorubicin in PLURONIC P-105 micelles (Micellar), and 5 μg/ml doxorubicin in PLURONIC P-105 micelles with ultrasound (Micelle/US).

FIG. 9 shows the effect of temperature and PLURONIC P-105 concentration on ruboxyl fluorescence intensity (ruboxyl concentration = 10 μg/ml): shaded, 25°C; hatched, 37°C; stippled, 42°C.

FIG. 10 shows Stern-Volmer plots for (a) ruboxyl and doxorubicin titration with iodide in PBS; (b) doxorubicin titration with iodide in 10 wt% PLURONIC P-105; and (c) doxorubicin titration with iodide in 20 wt% PLURONIC P-105 and

ruboxyl titration with iodide in 10 wt% and 20 wt % PLURONIC P-105; all treatments were at 37°C.

FIG. 11 shows the effect of ruboxyl encapsulation in PLURONIC P-105 on the drug uptake by HL-60 cells: fluorescence of HL-60 cell lysate normalized to the cell concentration as a function of PLURONIC P-105 concentration; ruboxyl concentration = 40 μg/ml, 1 hour.

FIG. 12 shows static quenching of doxorubicin fluorescence on doxorubicin intercalating into DNA in the absence (□) and presence (●) of PLURONIC P-105; doxorubicin concentration = 10 μg/ml, DNA concentration = 11 μg/ml.

FIG. 13 shows that doxorubicin encapsulation in PLURONIC micelles restricts drug intercalation into DNA: fraction of retained fluorescence on doxorubicin intercalation into DNA as a function of PLURONIC P-105 concentration; doxorubicin concentration = 10 μg/ml, DNA concentration = 11 μg/ml.

FIG. 14 shows doxorubicin fluorescence in the lysates of HL-60 cells incubated without sonication (shaded) or sonicated (hatched) with doxorubicin (20 μg/ml) for 1 hour, normalized to the cell concentration.

FIG. 15 shows uptake (0 to 120 minutes) and retention (120 to 260 minutes) of doxorubicin in HL-60 (◇) and HL-60/R (△) cell; error bars represent standard deviations (n=3) around the mean, but are not shown if smaller than the symbol size.

FIG. 16 shows uptake and retention of doxorubicin in HL-60 cells: (□) no ultrasound treatment, (●) ultrasound treatment at 3.6 W/cm2 and 80 kHz for 120 minutes followed by a rinse (arrow) and discontinuing of ultrasound treatment; error bars represent standard deviations.

FIG. 17 shows uptake and retention of doxorubicin in HL-60/R cells: (□) no ultrasound treatment, (●) ultrasound treatment at 3.6 W/cm2 and 80 kHz, (■) no ultrasound treatment for 120 minutes followed by ultrasound treatment at 3.6 W/cm2 and 80 kHz, and (○) ultrasound treatment at 3.6 W/cm2 and 80 kHz for 120 minutes followed by a rinse (arrow) and discontinuing of ultrasound treatment; error bars represent standard deviations.

FIG. 18 shows uptake and retention of ruboxyl in HL-60/R cells: (□) no ultrasound treatment, ( ) ultrasound treatment at 3.6 W/cm2 and 80 kHz, (■) no

ultrasound treatment for 120 minutes followed by ultrasound treatment at 3.6 W/cm2 and 80 kHz, and (○) ultrasound treatment at 3.6 W/cm2 and 80 kHz for 120 minutes followed by a rinse (arrow) and discontinuing of ultrasound treatment; error bars represent standard deviations.

FIG. 19 shows percent survival of cells as determined by the MTT assay versus doxorubicin concentration: (□) HL-60 cells, no ultrasound treatment; (■) HL-60 cells, ultrasound treatment at 3.6 W/cm2 and 80 kHz; (○) HL-60/R cells, no ultrasound treatment; and (●) HL-60/R cells, ultrasound treatment at 3.6 W/cm2 and 80 kHz.

FIG. 20 shows percent survival of cells as determined by the MTT assay versus ruboxyl concentration: (□) HL-60 cells, no ultrasound treatment; (■) HL-60 cells, ultrasound treatment at 3.6 W/cm2 and 80 kHz; (○) HL-60/R cells, no ultrasound treatment; and (●) HL-60/R cells, ultrasound treatment at 3.6 W/cm2 and 80 kHz.

DETAILED DESCRIPTION

Before the present composition and method for drug delivery are disclosed and described, it is to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to a composition containing "a drug" includes a mixture of two or more drugs, reference to "a copolymer" includes reference to one or more of such copolymers, and reference to "a micelle" includes reference to a mixture of two or more micelles.

As used herein, "effective amount" means an amount of a drug or pharmacologically active agent that is nontoxic but sufficient to provide the desired

local or systemic effect and performance at a reasonable benefit/risk ratio attending any medical treatment.

As used herein, "administering" and similar terms mean delivering the composition to the individual being treated such that the composition is capable of being circulated systemically to the parts of the body where the drug is to act, such as the site of a tumor. Thus, the composition is preferably administered to the individual by systemic administration, typically by subcutaneous, intramuscular, or intravenous administration, or intraperitoneal administration. Injectables for such use can be prepared in conventional forms, either as a liquid solution or suspension or in a solid form suitable for preparation as a solution or suspension in a liquid prior to injection, or as an emulsion. Suitable excipients include, for example, water, saline, dextrose, glycerol, ethanol, and the like; and if desired, minor amounts of auxiliary substances such as wetting or emulsifying agents, buffers, and the like can be added.

The EPR technique has been used previously to screen various members of the PLURONIC family of triblock copolymers to determine their micellization behavior (N. Rapoport & K. Caldwell, 3 Colloids and Surfaces B: Biointerfaces 217-228 (1994)). A lipophilic spin probe, 16-doxyl stearic acid (DSTA) was used to report the hydrophobicity of the micelle core and the solubilization efficiency of PLURONIC micelles. PLURONIC P-105 was found, depending on the concentration, to exhibit three regions on a phase diagram corresponding to unimers, loose aggregates, and dense micelles. At the onset of dense multimolecular micelle formation, the PLURONIC solubilization efficiency for lipophilic substances increased dramatically (FIG. 1).

The EPR technique was used to determine the characteristics of other PLURONIC copolymers. Neither PLURONIC F-68 nor PLURONIC F108 formed micelles having hydrophobic cores, and their solubilization efficiency for DSTA remained very low even at concentrations of 20 wt%. Liquid PLURONIC copolymers L-10 and L-92 formed micelles with hydrophobic cores. These micelles, however, manifested acute toxicity on HL-60 cells. Therefore, PLURONIC P-105 is a preferred triblock copolymer according to the present invention.

Two anti-cancer drugs are used in the presently described experiments (FIG. 2). Doxorubicin (also known as adriamycin) is widely used in clinical practice as a chemotherapeutic agent. It is an intercalating drug that stacks between paired bases in DNA. A strong drug-DNA interaction is critical for the drug's cytotoxic effect. Like other anti-cancer drugs of the anthracycline family, however, doxorubicin is cardiotoxic due to the induced production of active oxygen radicals (W.B. Pratt et al., in The Anticancer Drugs 155-182 (Oxford Univ. Press 1994); N.M. Emanuel et al., 53 Russian Chem. Rev. 1121-1138 (1984); J.H. Doroshow, Role of Reactive Oxygen Production in Doxorubicin in Cardiac Toxicity (Martinus Nijhoff Pub. 1988)).

A paramagnetic analog of doxorubicin, i.e. ruboxyl, has a paramagnetic Tempo-type nitroxide radical (1-oxo-2,2,6,6-piperidone-4-hydrazone) conjugated to doxorubicin (FIG. 2). The nitroxide moiety in position 14 serves as a radical trap. Ruboxyl is both fluorescent and paramagnetic, which provides for fluorescence and EPR spectroscopy to be used independently of drug uptake, distribution, and metabolism. This makes ruboxyl a powerful research tool. The anti-tumor activity of ruboxyl on models of leukosis, La, P-388, and L-1210, inoculated on mice and on solid tumors in rats has been reported to be high (M. Yokoyama, Site Specific Drug Delivery Using Polymeric Carriers, in Advances in Polymeric Systems for Drug Delivery (1994)). In clinical trials the drug was found effective against breast and colon carcinomas and bone sarcoma, and cardiotoxicity was reduced.

Example 1

Solubilization of Doxorubicin in PLURONIC P-105

The solubilization of doxorubicin in triblock copolymer solutions of concentrations ranging from 0.1 to 20 wt% was studied by fluorescence quenching experiments. A doxorubicin solution in PBS or PLURONIC P-105 was placed in a cuvette and the initial fluorescence was measured. Aliquots of quenching solutions were then added. In this example, iodide, added as KI mixed with the antioxidant $N_2S_2O_3$, was used as a quenching agent (B. Baleux & J. Shampetier, 274 C. R. Acad. Sci. Paris 1617-1620 (1972)). Ionic quenchers are charged and hydrated, and consequently should only be able to quench free doxorubicin adsorbed on surface

residues or in a relatively hydrophilic environment. Fluorescence was then determined in the presence of the quencher. All of these fluorescence experiments were carried out at 25°C.

PLURONIC P-105, with an average molecular weight of about 6500, was obtained from BASF Corporation. The average number of monomer units in polyethylene oxide (PEO) and polypropylene oxide (PPO) segments were 37 and 56, respectively. The copolymer was dissolved at various concentrations (0.1-10 wt%) in RPMI medium, and the solutions obtained were sterilized by filtration through a 0.2 μm filter.

The data were then used to generate a Stern-Volmer plot (G.S. Karczmar & T.R. Tritton, 557 Biochim. Biophys. Acta 306-319 (1979); G.S. Kwon et al., 2 Colloids Surfaces B: Biointerfaces 429-434 (1994); G.S. Kwon et al., 12 Pharm. Res. 192-195 (1995); G.S. Kwon & K. Kataoka, 16 Adv. Drug Delivery Rev. 295-309 (1995); all of which are hereby incorporated by reference). The Stern-Volmer equation is: $F_0/F = 1/K_{SV}[I^-]$, where F_0 and F are the measured fluorescence intensities in the absence and presence of iodide at concentrations $[I^-]$, and K_{SV} is the collisional quenching constant. The experimentally determined quenching constant is equal to the product of the excited state life time in the absence of quencher (t_0) and the bimolecular rate constant for collision between the reactants (k): $K_{SV} = kt_0$.

FIG. 3 shows the Stern Volmer plots of 5 μg/ml doxorubicin and PLURONIC P-105 at five concentrations ranging from 0 to 20 wt%. Linear plots were obtained for all five cases. A K_{SV} of 27.8 M^{-1} was obtained for free doxorubicin, which is consistent with the reported literature value (G.S. Karczmar & T.R. Tritton, 557 Biochim. Biophys. Acta 306-319 (1979)). The inset of FIG. 3 shows a plot of the slopes of the Stern-Volmer plots as a function of the PLURONIC P-105 concentration. The decrease in the slopes with increasing PLURONIC P-105 concentration indicates that upon the addition of surfactant, the drug is less accessible to iodide, suggesting that doxorubicin partitions to the hydrophobic microdomains of the surfactant and is only somewhat protected from the iodide. The slopes of data at 0.1% (unimers) and at 1.0% (loose aggregates) are only slightly lower than in a surfactant-free solution. The K_{SV} of 10% and 20% solutions are much lower and have nearly the same values, which shows that these concentrations,

corresponding to the formation of dense micelles, are much more effective in protecting doxorubicin from the iodide. The slopes of the data at 0.1 wt% (unimers) and at 1.0 wt% (loose aggregates) are only slightly lower than in a surfactant-free solution. The K_{SV}'s of 10 wt% and 20 wt% solutions are much lower and have nearly the same values, which shows that these concentrations, corresponding to the formation of dense micelles, are much more effective in protecting doxorubicin from the iodide. Even at these high PLURONIC P-105 concentrations, iodide showed a weak quenching of doxorubicin, indicating the presence of some free drug in the solutions. It should be noted that a decrease in the K_{SV} values with an increase in PLURONIC P-105 concentration may be due to changes in both k and t_0. The change in the polarity of the microenvironment after doxorubicin incorporation in the micelle results in an increase in the fluorescence of the drug. Therefore, doxorubicin has enhanced fluorescence life times. Unless the doxorubicin lifetimes within the micelles are determined, individual contributions to K_{SV} cannot be determined. However, in the event of a decrease in either k or t_0, both correspond with the entrapment of doxorubicin in micelles.

The other contributory factors to quenching are temperature and viscosity. All the fluorescence experments were carried out at 25°C. The micelle formation of PLURONIC P-105 solutions is very sensitive to the temperature (P. Alexandridis et al., 27 Macromolecules 2414-2425 (1994)). The hydrophobic part (i.e. the PPO) is responsible for micellization due to diminishing hydrogen bonding between water and PPO with increasing temperature. At a given concentration, the multimolecular micelles are formed at temperatures exceeding the critical micelle temperature, which is 37°C in this case. It is quite probably that at 25°C the micelles are not quite as dense (compared to 37°C) and doxorubicin may not completely localize within the hydrophobic cores, resulting in some quenching of drug by the iodide. Viscosity measurements showed that there is no significant difference in viscosities of PBS, 0.1 wt% PLURONIC P-105 solution, and 1.0 wt% PLURONIC P-105 solution, though viscosity does increase at higher concentrations (i.e. 10 wt% and 20 wt%) of PLURONIC P-105. Despite the same viscosities, the slopes of Stern-Volmer plots for doxorubicin associated with unimers (0.1 wt%) and loose aggregates (1.0 wt%) are significantly less than the slope of doxorubicin in PBS,

indicating that a decrease in slope cannot be attributed to increase in viscosity only. Therefore, lower K_{SV} values for 10 wt% and 20 wt% PLURONIC P-105 solutions cannot be attributed completely to an increase in viscosity of the solution. These results suggest that at higher concentrations of PLURONIC P-105, doxorubicin has greater compatibility with the hydrophobic cores of the PEO-PPO-PEO micelles.

Example 2

Cytotoxicity of PLURONIC P-105 Copolymer and Ultrasound

HL-60 promyelocytic cell lines were cultured in RPMI medium supplemented with 20% fetal calf serum, 2 mM L-glutamine, 7.5% sodium bicarbonate, and 50 μg/ml gentamicin at 37°C. Cultures were maintained in 75 ml plastic tissue culture flasks and kept in an incubator at 37°C in humidified air containing 5% CO_2.

Ultrasound was generated by a Sonicor SC 100 sonicating bath operating at a frequency of 80 kHz (Sonicor Instruments, Copiaque, NY). The ultrasonic bath was maintained with about 2.5 liters of water at 37°C using a thermostat. The power density delivered by the bath was controlled by adjusting the input voltage with a variac and was measured using a hydrophone (Z. Qian, MS Thesis, Brigham Young University (1996)).

FIG. 4 shows the growth curve of HL-60 cells preincubated for 6 h with 0.1, 1.0, and 10 wt% PLURONIC P-105 solutions at 37°C. The figure shows the effect of increasing concentration of PLURONIC P-105 on the growth of the cells. At low concentrations, there is not much difference between the growth patterns of the control and 0.1 wt% PLURONIC P-105 treated cells. At higher concentrations, however, the cell division takes place rather slowly, but eventually grows to the same order of magnitude s the control.

FIG. 5 shows the dual effects of PLURONIC P-105 and ultrasonication. The cells subjected to ultrasound alone do not show any increase in lag phase, but the cells preincubated with the PLURONIC P-105 under sonication exhibited a different growth pattern. At a concentration of 10 wt% PLURONIC P-105 combined with sonication, the cells show a very prolonged lag phase.

The effect of preincubation of PLURONIC P-105 and ultrasound on cells was studied for different intervals of time ranging from 1 to 24 hours. It was apparent from the data (not shown) that at higher concentration (10 wt%) of the surfactant, incubation time of up to 6 h did not inhibit the cell growth, as in all cases the cell population grew to the same order of magnitude. Though the effect of ultrasound prevails at all incubation times (i.e. 1, 6, 12, and 24 hours), the most significant effect is seen at 12 or more hours preincubation time with 1 wt% or greater PLURONIC P-105. Above 1 wt% concentration, the effect of surfactant has a much stronger influence than sonication on the inhibition of cell growth.

FIG. 6 shows the survival curves of the cells treated with surfactant with and without ultrasound. Only two preincubation times are shown. The concentration of PLURONIC P-105 producing the 50% inhibition of cell growth appears to be low, but it should be noted that the combined sonication and surfactant treatment seemed to arrest the growth of the cells initially. These cell counts were taken at 72 hours. At this time point, the untreated cells were already in the log phase of growth, but the treated cells were still in the lag phase.

These results indicate that the inhibition of cell growth in the initial phase of the growth curve inflicted by higher concentrations of PLURONIC P-105 within 12 hour exposure are not terminally toxic s the normal cell growth is restored after this period. Incubation times of 12 hours and more, however, produce considerable cytotoxic effects. Sonication seems to enhance this effect, most likely by destabilizing the cell membrane. The toxicity associated with high concentrations of PLURONIC P-105 can be attributed to the behavior of non-ionic surfactants, since they are known to act in different ways at different concentrations. At high concentration, i.e. above the critical micelle concentration (CMC), they solubilize the biological membranes by forming mixed micelles of surfactant, phospholipids, and integral proteins. At low concentrations, i.e. below the CMC, they may bind to the hydrophobic regions of the membrane proteins without disrupting the membrane.

Example 3

Comparative Cytotoxic Effects with Doxorubicin

The growth inhibition method was used to determine the cytotoxicity of PLURONIC P-105. HL-60 cells were subjected to incubation or ultrasonication with the copolymer for various intervals of time at 37°C. Exposure was terminated by removing the PLURONIC P-105-containing medium by rinsing twice with fresh medium and subsequently culturing the cells for 4 days. Cell counts were taken at various times post-treatment. Drug treatments were for 1 hour at 37°C on cells in exponential growth phase at 10^6 cells/ml density. In the cytotoxicity assay with the drug, the cells were subjected to four different treatments: (1) free doxorubicin, (2) free doxorubicin with ultrasound, (3) doxorubicin in micelles, and (4) doxorubicin in micelles with ultrasound. After treatment, the cells were centrifuged at 500 x *g*, washed twice, resuspended in drug-free medium, and cultured for 4 days. Immediately after these exposures, no early cell death was detected. The cells were counted in a hemacytometer, and viability was determined by the dye exclusion test (A. Rahman et al., 84 J. Nat'l Cancer Inst. 1909-1915 (1992), hereby incorporated by reference). IC_{50} was defined as the concentration of doxorubicin resulting in 50% survival of the cells after 96 hours compared with the control.

FIG. 7 shows the survival curves of 1 hour exposure to free doxorubicin, free doxorubicin and ultrasound, doxorubicin in micelles (10 wt% PLURONIC P-105), and doxorubicin in micelles and ultrasound. The data shown in FIG. 7 are the averages of five replicate experiments. The concentration of doxorubicin that caused 50% inhibition of cell growth (IC_{50}) after 1 hour exposure with free doxorubicin, free doxorubicin with ultrasound, micelles, and micelles with ultrasound were 2.35 μg/ml, 0.9 μg/ml, 1.25 μg/ml, and 0.19 μg/ml, respectively. Based on these values (inset of FIG. 7), the drug delivered through micelles in combination with ultrasound is 12-fold more effective in inhibiting the cell growth than the free drug. There is not a significant difference between the effect of free drug with ultrasound and micellar drug exposures. The combination of ultrasound and drug in micelles appears to have at least an additive effect on the cells. In some experiments, the HL-60 cells were treated separately to PLURONIC -105 and ultrasound. Though the power density

at which the ultrasound was delivered was quite high (3.6 W/cm^2), exposure of this power level for 1 hour did not produce any toxic effects on the cells.

Example 4

Cellular Accumulation of Doxorubicin

The Pluronic uptake by the HL-60 cells was measured by its depletion in the culture using a well developed iodometric technique (B. Baleux & J. Shampetier, 274 C. R. Acad. Sci. Paris 1617-1620 (1972), hereby incorporated by reference). The cells were subjected to the treatments described in Example 3 for 1 hour. After rinsing and washing twice with PBS, the cells were suspended in cold PBS and centrifuged again. The cell pellets were lysed by suspending in 0.5 ml of 2% SDS solution and then sonicating for 10 minutes. The fluorescence of the lysates was read using a spectrofluorometer at an excitation wavelength of 488 nm and an emission wavelength of 590 nm. The relative fluorescence intensity of the untreated cells was subtracted from that of the drug-treated cells. The doxorubicin accumulation was quantified by measuring the fluorescence intensity, and the uptake was expressed as relative fluorescence intensity units.

FIG. 8 illustrates the accumulation of doxorubicin in HL-60 cells following treatment with 5 μg/ml free drug, free drug and ultrasound, micellar drug, and micellar drug with ultrasound for 1 hour. The highest uptake of doxorubicin was observed when the drug was delivered through micellar solution under the influence of sonication. The lowest uptake of drug was observed from micellar solution (10 wt% PLURONIC P-105). Sonication seemed to increase the drug uptake with both free and micellar solutions. With no sonication, the cells accumulate less doxorubicin from micelles than they accumulate from free doxorubicin (without PLURONIC P-105). This indicates that micelles effectively sequester doxorubicin from the HL-60 cells until activated by ultrasound.

The reduced IC_{50} values with doxorubicin in micelles with ultrasound and free doxorubicin with ultrasound correspond to the observed higher uptake of the drug. There is a reduced IC_{50} with doxorubicin in PLURONIC P-105 without ultrasound treatment, but the uptake studies show less doxorubicin accumulation by the cells from the micellar solution. The reason for a reduced IC_{50} despite a lower

uptake of drug is not clear. It cannot be explained by an additive cytotoxic effect of the copolymer, since under the conditions studied, the copolymer alone did not affect the cell viability. Cytotoxicity data of PLURONIC P-85 on human ovarian carcinoma cells (V.Y. Alakhov et al., 7 Bioconjugate Chem. 209-216 (1996)) also suggest that in the presence of copolymer, lower amounts of the drug that intercalated with cellular DNA caused substantially higher cytotoxic effects. The important question here is whether the copolymer is take up by the cells together with doxorubicin, and in what form (micellar or unimeric) it is being transported into the cells. To understand this process, the uptake of PLURONIC P-105 by the cells over concentrations ranging from below the CMC to concentrations above the CMC were studied. It was found that PLURONIC P-105 is not all taken up by the cells at very low concentrations, but cells begin to sorb the copolymer at concentrations around the CMC and above. This result suggests the interaction of cells with the drug encapulated in micelles rather than drug associated with unimers.

The most common and accepted form of cancer treatment, chemotherapy, is often limited by its deleterious side effects on normal tissues and a host of other problems, all of which compromise the patient's health. Therefore, a desirable improvement would be to reduce the dosage or frequency of drug administration by improving the effectiveness of drugs at the targeted site. It is shown herein that the combination of ultrasound and micellar drug carriers can lower the effective dosage of an anti-cancer drug, which provides a way to reduce the toxic side effects associated with high doses of chemotherapeutic drugs. The interaction of anti-cancer drugs with normal tissues can be circumvented by encapsulating the drug in polymeric micelles. PLURONIC P-105 is a non-toxic copolymer at concentrations much higher than the CMC and has no recognition with RES, although its cytotoxicity on normal cells has yet to be determined. The use of ultrasound is advantageous in the sense that ultrasound is a non-invasive technique. Ultrasound can be focused at selected depths in soft tissue throughout the body. This approach is capable of depositing large amounts of ultrasonic energy into deep tumors. By taking advantage of the non-invasive technique of ultrasound and creating non-toxic micellar drug carriers, a new approach to drug targeting is provided.

Example 5

Micellization Using Ruboxyl and Doxorubicin as Fluorescent Probes

The anthraquinone moieties of ruboxyl and doxorubicin are inherently fluorescent, which makes it possible to use them as fluorescent probes. The fluorescence of both roboxyl and doxorubicin is quenched by collisions with water molecules. When ruboxyl and doxorubicin are prevented from colliding with water, their fluorescence increases about 3-fold. For example, at ruboxyl concentrations of 20 μg/ml, fluorescence intensity is 8200 (in arbitary units) in PBS and 29,800 in ethanol. This phenomenon was used to study the micellization process of various members of the PLURONIC family.

Technical fluorescence emission spectra were recorded over a temperature range of 25-42°C using a photon counting spectrofluorometer (ISS, model PC-1, Champaign, IL). As could be expected, the ruboxyl fluorescence increased dramatically upon the onset of dense micelle formation in PLURONIC P-105 solutions (FIG. 9). Copolymer concentrations corresponding to the onset of dense micelle formation decreased with increasing temperature.

The solubilization efficiency of PLURONIC micelles for lipophilic compounds was monitored by the quantitative EPR technique using DSTA as a spin probe. PLURONIC solutions of various concentrations were incubated with DSTA powder at room temperature for 15 minutes under constant shaking. The non-stabilized fraction of the probe was separated by centrifugation. EPR spectra were collected from supernatants. The intensities (double integrals) of EPR spectra were compared to those of standard solutions.

The EPR spectra were recorded at room temperature with an X-band Bruker ER-200 SRC EPR spectrometer. Incident microwave power was set to 0.5-2 mW to avoid saturation. A modulation frequency of 100 kHz was used, and the modulation amplitude was typically a quarter of a linewidth.

EPR and fluorescence data were in good agreement in terms of copolymer concentration corresponding to the onset of dense micelle formation.

The additivity model may be used to analyze fluorescence intensity data:

$$I_{exp} = a_m f_m + a_s(1-f_m)$$

where a_m and a_s are quantum yields of probe fluorescence in hydrophobic and hydrophilic environments, respectively, and f_m is the fraction of the probe located in the hydrophobic environment, i.e. in the hydrophobic micelle core. Free drug in solution and drug molecules associated with loose, water-penetrated PLURONIC P-105 aggregates are located in a hydrophilic environment.

Based on this model, the present data indicated that at 37°C (the temperature of drug incubation with living cells) and in 1 wt% PLURONIC P-105 solutions, about 45% of the drug was localized in the hydrophobic environment, and in 10 wt% PLURONIC P-105 solutions 100% of the drug was localized in the hydrophobic environment.

Other PLURONIC copolymers having longer PEO blocks (F-88, F-108, F-127) never formed dense micelles with hydrophobic cores, as evidenced from both fluorescence and EPR experiments.

Example 6

Drug Localization in PLURONIC Micelles

Ruboxyl and doxorubicin were introduced into PLURONIC P-105 micellar solutions from stock solutions in PBS or RPMI medium. Non-solubilized drug was removed by dialysis through a 1000 dalton cutoff membrane at 37°C.

To assess the accessibility of solubilized drug molecules to the external quencher, Stern-Volmer plots for dynamic fluorescence quenching were derived by measuring the decrease of fluorescence intensity upon injecting progressively increasing concentrations of KI into doxorubicin or ruboxyl solutions in PBS or PLURONIC P-105.

Ruboxyl and doxorubicin encapsulated in the inner core of PLURONIC P-105 micelles were effectively protected from interaction with substances that did not penetrate into the micelle core, e.g. iodide anion (which is an effective fluorescence quencher). Stern Volmer plots of doxorubicin and ruboxyl dynamic quenching by iodide are presented in FIG. 10, which demonstrates a significant decrease of Stern-Volmer quenching constants upon drug solubilization in dense PLURONIC micelles.

The Stern-Volmer quenching constant, K_{SV}, (slope of line) dropped about 4-fold upon ruboxyl encapsulation in PLURONIC micelles. The Stern-Volmer

constant is a product of flurophore-quencher collision constant (k_q) and the fluorescence lifetime τ. The fact that fluorescence intensity of doxorubicin and ruboxyl within PLURONIC micelles is much higher than in PBS solutions indicates that fluorescence lifetime in micelles is longer than in a molecular solution in PBS. Thus, the drop of K_{SV} observed upon ruboxyl and doxorubicin solubilization in PLURONIC micelles results from the decrease of drug-iodide collision constant due to the insertion of the drug into the interior of the micelle.

It is noteworthy that doxorubicin was somewhat more accessible than ruboxyl to collisions with iodide in micelles, reflecting deeper insertion of ruboxyl into the micelle core.

Example 7

Drug Loading and Release from PLURONIC Micelles

To study solute release from PLURONIC micelles, the partitioning of the solute between the micelles and the surface of polystyrene latex particles was investigated. A spin probe, DSTA, or the drug, ruboxyl, was solubilized in PLURONIC P-105 solutions of various concentrations. A suspension of polystyrene latex particles (average diameter 0.9 μm, 50 μl/ml) was incubated with 1 ml of DSTA or ruboxyl solution in PLURONIC P-105, and depletion of the probe in the supernatant was measured by the EPR (for DSTA) or fluorescence (for ruboxyl) technique upon polystyrene particle separation, according to the procedure of Example 6.

Upon introduction into micellar PLURONIC solutions, doxorubicin and ruboxyl were spontaneously transferred into the inner core of the PLURONIC micelles. Free drug (if any) was removed by dialysis.

An important question pertinent to this research was how tightly the solubilized drug was associated with PLURONIC micelles. To investigate this problem, ruboxyl adsorption on polystyrene latex particles was measured from molecular solutions of ruboxyl in PBS and from micellar PLURONIC solutions. Ruboxyl readily adsorbs onto polystyrene surfaces. About 90% of the drug is transferred onto polystyrene surface from ruboxyl solutions in PBS. PLURONIC micelles, however, compete for ruboxyl with polystyrene surfaces; only about 40%

of ruboxyl solubilized in PLURONIC micelles (20 wt% solutions of PLURONIC P-105) is transferred onto the polystyrene surface, the remainder being retained within the PLURONIC micelles.

Example 8

Effect of Drug Encapsulation in PLURONIC Micelles on the Intracellular Uptake by HL-60 Cells

Intracellular uptake of doxorubicin and ruboxyl was measured using a fluorescence technique wherein compounds were excited at 488 nm and technical emission spectra were recorded at 510-700 nm. Two sets of samples were studied, incubated, and sonicated. Ultrasound was generated by a Sonicor SC100 sonication bath operating at 70 kHz and 37°C. Power density was controlled by adjusting the input voltage and was measured with a hydrophone according to the procedure of Example 2.

For the first set of samples, the cells were incubated at 37°C with doxorubicin or ruboxyl, which were either dissolved in the RPMI medium or PBS, or the drugs were solubilized in PLURONIC P-105 solutions of various concentrations. For the second set of samples, the cells were sonicated by 70 kHz ultrasound at 37°C to assess the effect of ultrasound on the drug uptake from molecular and micellar solutions. After being incubated/sonicated with and without the drug, the cells were centrifuged, washed twice with cold PBS, resuspended in PBS, and the fluorescence spectra of cell suspensions were recorded. The fluorescence intensity of the untreated cells was subtracted from that of the drug-treated cells. Because drug fluorescence within the cells was substantially quenched, drug uptake was quantified by lysing the cells by incubating them with 1 wt% SDS solution for 1-2 hours at 37°C. This process transferred the drug from cellular components to SDS micelles. Calibration experiments showed a linear dependence of fluorescence intensity on ruboxyl and doxorubicin concentration in 1 wt% SDS solutions in the concentration range of interest. Upon the completion of cell lysis, fluorescence spectra of the lysates were recorded. To quantify the concentration of lysed cells, cell lysates were filtered through 0.2 μm filters, and their optical densities were measured by protein absorbance at 280 nm (OD 280 nm). Calibration

experiments showed a linear dependence of OD 280 nm on the concentration of lysed cells. The fluorescence intensity of lysates was normalized by OD 280 nm.

Drug sequestration in PLURONIC P-105 micelles caused a substantial decrease in drug uptake by HL-60 cells (FIG 11). These data are representative of numerous experiments on the uptake of ruboxyl and doxorubicin from PLURONIC P-105 micelles. The uptake of the drug was somewhat enhanced at a PLURONIC concentration of 0.1%, which is below the CMC for the formation of dense micelles, indicating that PLURONIC molecules in a unimeric form or in loose aggregates enhanced the permeability of cell membranes toward the drug. Drug uptake from dense PLURONIC micelles was substantially lower than that of a free drug, indicating that dense micelles inhibited drug interaction with the cells.

Ruboxyl and doxorubicin encapsulation in PLURONIC micelles restricted not only drug interaction with the cells, but also drug interaction with cell components, e.g. DNA, as manifested in FIGS. 12 and 13. The drop of fluorescence was lower when the drug was introduced from a micellar solution, indicating a lower drug-DNA interaction (FIG. 13).

Example 9

Effect of Ultrasound on Intracellular Drug Uptake

A decreased uptake of the drug solubilized in dense polymeric micelles requires a method to enhance drug intracellular uptake at the tumor site. Ultrasonication of the cells in the presence of micelle-encapsulated drugs can substantially enhance intracellular uptake of the drug. Typical results on drug accumulation within the cells are presented in FIG. 14. A similar effect is observed when drug uptake is measured by depletion from the incubation medium (data not shown). The investigation of doxorubicin cytotoxicity on HL-60 cells when the drug was delivered from molecular solutions (without PLURONIC) and from micellar solutions, with and without acoustic activation, has shown that the combination of micellar delivery and ultrasonication resulted in a substantial decrease of the effective drug dose. It is noteworthy that despite a decreased intracellular uptake of the micelle-encapsulated rug, its cytotoxicity was higher than that of a free drug, probably due to the cytotoxic effect of PLURONIC micelles on mitotic cells.

Example 10

Size of PLURONIC Micelles

Micelle size was measured by dynamic light scattering using a BI 200 Spectrometer from Brookhaven Instruments equipped with a He-Ne laser (632.5 nm) and a BI 2030 72-channel autocorrelator.

The size of PLURONIC micelles was measured at 37°C by photon correlation spectroscopy. For dense micells in 20 wt% PLURONIC P-105 solutions, micelle diameter was about 13 nm. Micelle diameter and the polydispersity of the system increased upon dilution of PLURONIC solutions.

Ruboxyl and doxorubicin intracellular uptake from solutions containing PLURONIC unimers or loose aggregates was slightly enhanced with that from the medium, apparently due to the effect of a polymeric surfactant on the permeability of cell membranes. In contast, ruboxyl and doxorubicin encapsulation in dense polymeric micelles substantially decreased drug uptake by the cells. In this case, the protective effect of drug solubilization in the dense hydrophobic micelle cores overcomes the effect of the polymeric surfactant on the cell membrane permeability. Decrease drug uptake from micelles is advantageous for preventing undesired drug interactions with normal cells.

The size of PLURONIC micelles at 37°C (13-15 nm), is advantageous for their long circulation in the blood without extravasation into normal tissues. On the other hand, micelles of this size are expected to extravasate in tumor sites due to the higher permeability of tumor blood vessels. Thus, the accumulation of the micelle-encapsulated drug in the tumor site might be expected.

Ultrasonication enhances drug uptake from PLURONIC micelles. Based on this finding, a new concept of a localized drug delivery may be developed, based on encapsulating a drug in stabilized micelles, administering the drug-containing micelles, and focusing ultrasound on the tumor.

There are two possible mechanisms of acoustically-enhanced intracellular uptake of the drug from micellar solutions: (1) acoustically-enhanced drug release from micelles and (2) acoustic effect on the permeability of cell membranes.

Example 11

A doxorubicin-resistant subline of HL-60 cells, hereinafter referred to as HL-60/R, was derived from HL-60 cells by continuous exposure to increasing concentrations of doxorubicin until the cells could grow in the presence of 1 μg/ml of doxorubicin. Before any experiments with this cell line were undertaken, the cells were maintained in drug-free medium for a minimum of three passages.

Cells were incubated or ultrasonicated in the exponential growth phase at 10^6 cells/ml density in complete RPMI medium without or wtih doxorubicin or ruboxyl at the concentration of 5 μg/ml. To measure drug uptake (both doxorubicin and ruboxyl), aliquots of cells were obtained at 0, 15, 30, 60, 90, and 120 minutes of incubation or ultrasonication. After rinsing and washing twice with sterile PBS, the cells were centrifuged again. The cell pellets were immediately lysed by adding 2 ml of 2% SDS solution. The lysates were then incubated at 37°C for 1 hour and then ultrasonicated for 10 minutes. The drug uptake was quantified by measuring the fluorescence intensity of lysates using a spectrofluorometer at an excitation wavelength of 488 nm and an emission wavelength of 590 nm. The uptake and retention were expressed as relative fluorescence intensity units. The fluorescence intensity was normalized to the cell mass by dividing the intensity at 590 nm by the optical density of filtered lysates at 280 nm. For retention studies, following 120-minute uptake phase, each sample was washed twice with PBS and resuspended in drug free medium. Then, the samples were either incubated or ultrasonicated for additional 120 minutes under the same conditions as for the uptake studies. Aliquots of cells were taken at 15, 30, 60, 90, and 120 minutes after rinsing, and the uptake was quantified as described above.

FIG. 15 compares the uptake and retention of doxorubicin by the parent and resistant cell lines. The HL-60/R cells showed slower doxorubicin uptake, and when resuspended in drug-free medium, there was a rapid decrease in intracellular doxorubicin concentration. These data are consistent with an increased efflux mechanism in the resistant cell line. During incubation in drug-free medium, the doxorubicin concentration in both cell lines quickly reached a plateau value, which presumably corresponds to irreversibly bound drug. Note that the concentration of

irreversibly bound drug is much lower in the resistant cell line, while nearly all the drug is bound in the parent cell line.

FIGS. 16 and 17 show the effect of ultrasound on the uptake and retention of doxorubicin in HL-60 and HL-60/R cells, respectively. The uptake is higher when given in combination with ultrasound. The main differences in these figures is the level of retention with and without ultrasonication. In the HL-60 cell line, nearly all the doxorubicin is irreversibly bound, and ultrasound produced only a slight effect on retention. In the HL-60/R cells, however, the application of ultrasound during the uptake phase resulted in a much larger fraction of irreversibly bound drug, approaching the level observed in HL-60 cells. During the retention phase of the experiments with HL-60/R cells, the application of ultrasound, either before or during retention, had no significant effect on the level of retained drug (FIG. 17).

Similar observations were obtained for both of these cell lines exposed to ruboxyl. FIG. 18 shows the effect of ultrasound on the uptake and retention of ruboxyl in the HL-60/R cell line. Again, the rate of uptake and the level of retained drug was higher when exposed to ultrasound during the uptake phase.

Example 12

Cytotoxicity

Cytotoxicity assays were performed in quadruplicate with HL-60 and HL-60/R cells. The cells were either incubated or ultrasonicated for 2 hours at 37°C with various drug concentrations. The treatment was terminated at 2 hours by washing the cells and resuspending them in fresh medium. These pretreated cells were then plated in a 96-well microtiter plate and recultured for 72 hours. The drug cytotoxicity activity was evaluated using the MTT assay as described in W. Priebe & R. Perez-Soler, 60 Pharmac. Ther. 215 (1993) (hereby incorporated by reference). Cells also received exposure to ultrasound for 2 hours without drug and were similarly cultured and assayed. There was no cytotoxic effect caused by the ultrasound without drug.

The cytotoxicity assays of HL-60 and HL-60/R cells with doxorubicin and ruboxyl with or without ultrasound are should in FIGS. 19 and 20. The first point of interest in these data is that the HL-60/R cells that developed resistance to

doxorubicin became resistant to ruboxyl also. As shown in FIG. 19, the IC_{50} for HL-60 cells with doxorubicin was about 0.1 μg/ml, and ultrasonication reduced this value to around 0.005 μg/ml. Since ultrasonic exposure without drug is not cytotoxic, there appeared to be a synergistic effect wherein ultrasound rendered the cells much more sensitive to doxorubicin. The IC_{50} for HL-60R cells with doxorubicin was much higher, around 4 μg/ml. Most importantly, the application of ultrasound on the resistant cell line reduced the IC50 to about the same as that of the HL-60 cell line without ultrasound.

Similar observations were found for ruboxyl, as shown in FIG. 20. Again, ultrasound reduced thte IC_{50} of the HL-60 cell line, and reduced the IC_{50} of the HL-60/R cell line to about the same as that of the HL-60 cell line without ultrasound.

The results presented in the above examples showed significant drug efflux from doxorubicin-resistant cells upon incubation in a drug-free medium, suggesting that the drug was localized in the cytoplasm, not bonded to DNA and accessible to the action of the P-gp pump. In contrast, when the drug was given with ultrasound, subsequent drug efflux from resistant cells was substantially reduced, suggesting that a larger fraction of the intracellular drug was irreversibly bound to DNA, i.e. not accessible to the efflux pump. It was shown above that ultrasonication enhances cell membrane permeability and intracellular drug uptake. This would result in a higher intracellular drug accumulation if the rate of drug efflux was not affected by ultrasound. These results using ultrasonication in both the accumulation and efflux phases showed that ultrasonication indeed did not significantly affect the efflux rate, which explains the higher drug uptake by insonated cells.

The substantial decrease in efflux from ultrasonicated drug-resistant cells suggests that there is a difference in the intracellular drug distribution for insonated and non-insonated cells, the DNA-bound fraction of the drug being higher in ultrasonicated cells.

In the present studies, the fluorescence intensities of cells were compared with those of cell lysates for ruboxyl-containing non-ultrasonicated and ultrasonicated cells. These data (Table 1) show that despite a higher overall drug uptake by ultrasonicated cells (characterized by fluorescence intensity of cell

lysates), the intracellular drug fluorescence in ultrasonicated cells is quenched much more than that in non-ultrasonicated cells. This indicates that a larger portion of the drug is bound to DNA in ultrasonicated cells. This is true for both sensitive and resistant cells, though the effect in sensitive cells is more pronounced.

Table 1

Treatment	Fluorescence of cells*	Fluorescence of lysates*	F_L/F_C
HL-60, non-sonicated	8.8	22.3	2.5
HL-60, ultrasonicated	2.4	29	12
HL-60/R, non-sonicated	6.5	12.7	1.95
HL-60/R, ultrasonicated	3.0	15.6	5.2

* Expressed in arbitrary units.

The fluorescent properties of doxorubicin and ruboxyl allowed a determination of their distribution within cells. For example, fluorescence from ruboxyl and doxorubicin are quenched when these drugs are intercalated in DNA. In contrast, fluorescence is enhanced when drugs partition into phospholipid membranes. Also, fluorescence intensity is substantially enhanced when drug-DNA complexes are destroyed by SDS treatment, which happens upon cell lysis. It has been discovered that the intracellular fluorescence of ruboxyl in HL-60 cells was much higher than that of doxorubicin, the difference decreasing upon cell lysis. This suggested that in contrast to doxorubicin, which was predominantly bound to DNA, ruboxyl partitioned between DNA and cell membranes. Ruboxyl fluorescence in non-lysed cells is caused predominantly by the drug localized in cell membranes, since is would be quenched if it were all in drug-DNA complexes. In cell lysates, drug-DNA complexes are destroyed, and drug is localized in the core of SDS micelles, which causes fluorescence enhancement in comparison with aqueous drug environment. In model experiments, measured fluorescence intensities were found

to be nearly the same for ruboxyl in 1% SDS solution as in a phospholipid bilayer. Because of the similar fluorescence, it was possible to estimate the fraction of the DNA-bound drug. In non-insonated HL-60 cells this fraction was 60%. In ultrasonicated HL-60 cells it increased to 92%. The values for non-insonated and ultrasonicated HL-60/R cells were 49% and 81%, respectively.

These results show that ultrasonication during cell incubation with ruboxyl caused substantially enhanced drug partitioning into DNA. This could result, for instance, from enhanced drug transport through the nuclear membrane. Such enhance drug intercalation into DNA may account for the reversal of drug resistance caused by ultrasound. These results are evidence that ultrasound can be used for attacking multiple drug resistance (MDR) cells, which owe their resistance to increased drug efflux.

CLAIMS

We claim:

1. A method for delivery of a drug to a selected site in a patient comprising the steps of:

(a) administering to said patient a composition comprising a micellar drug carrier having a hydrophobic core and an effective amount of said drug disposed in said hydrophobic core; and

(b) applying ultrasonic energy to said selected site such that said drug is released from said hydrophobic core to said selected site.

2. The method of claim 1 wherein said micellar drug carrier is an ABA-triblock copolymer.

3. The method of claim 2 wherein said ABA-triblock copolymer is a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer.

4. The method of claim 3 wherein said poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer has a molecular weight of about 6500.

5. The method of claim 1 wherein said drug is hydrophobic.

6. The method of claim 5 wherein said hydrophobic drug is an anthracycline.

7. The method of claim 6 wherein said anthracycline is doxorubicin.

8. The method of claim 6 wherein said anthracycline is ruboxyl.

9. A composition for delivery of a drug to a selected site in a patient comprising micellar drug carrier having a hydrophobic core and an effective amount of said drug disposed in said hydrophobic core.

10. The compositions of claim 9 wherein said micellar drug carrier is an ABA-triblock copolymer.

11. The composition of claim 10 wherein said ABA-triblock copolymer is a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer.

12. The composition of claim 11 wherein said poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer has a molecular weight of about 6500.

13. The composition of claim 9 wherein said drug is hydrophobic.

14. The composition of claim 13 wherein said hydrophobic drug is an anthracycline.

15. The composition of claim 14 wherein said anthracycline is doxorubicin.

16. The composition of claim 14 wherein said anthracycline is ruboxyl.

17. A method for enhancing uptake of a drug by cells at a selected site in a patient comprising the steps of:

(a) administering to said patient a composition comprising a micellar drug carrier having a hydrophobic core and an effective amount of said drug disposed in said hydrophobic core; and

(b) applying ultrasonic energy to said selected site such that said drug is released from said hydrophobic core and taken up by said cells.

18. The method of claim 17 wherein said micellar drug carrier is an ABA-triblock copolymer.

19. The method of claim 18 wherein said ABA-triblock copolymer is a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer.

20. The method of claim 19 wherein said poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer has a molecular weight of about 6500.

21. The method of claim 17 wherein said drug is hydrophobic.

22. The method of claim 21 wherein said hydrophobic drug is an anthracycline.

23. The method of claim 22 wherein said anthracycline is doxorubicin.

24. The method of claim 22 wherein said anthracycline is ruboxyl.

25. A method for reducing side effects in a patient from administration of a drug comprising the steps of:

(a) administering to said patient a composition comprising a micellar drug carrier having a hydrophobic core and an effective amount of said drug disposed in said hydrophobic core; and

(b) applying ultrasonic energy to said patient such that said drug is released from said hydrophobic core.

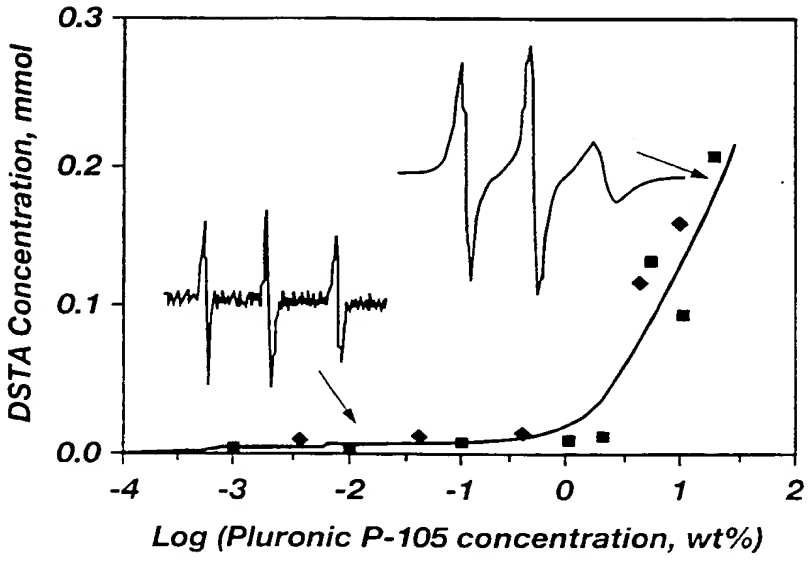
0.3
0.2
0.1
0.0
DSTA Concentration, mmol
-4
-3
-2
-1
0
1
2
Log (Pluronic P-105 concentration, wt%)

Fig. 1

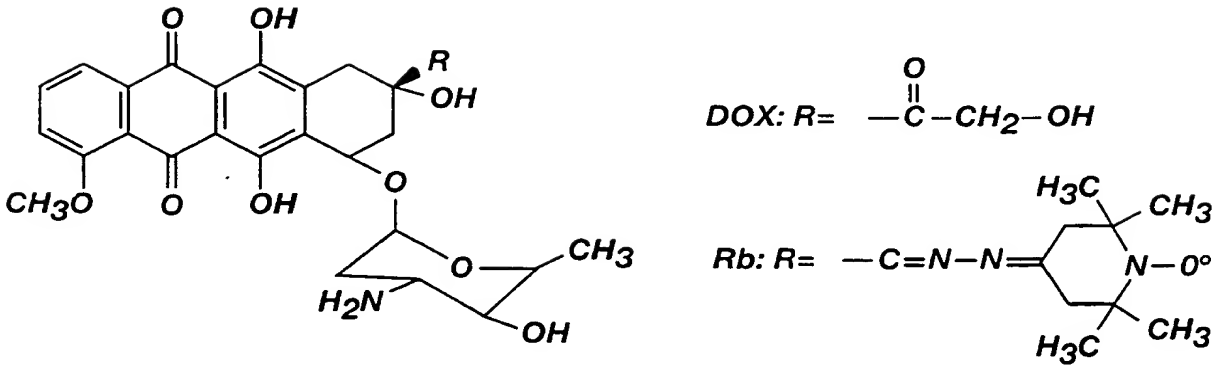
O
OH
R
OH
CH3O
O
OH
O
O
CH3
H2N
OH
DOX: R=
O
—C—CH2—OH
Rb: R=
—C=N—N=
H3C
CH3
N—O°
H3C
CH3

Fig. 2

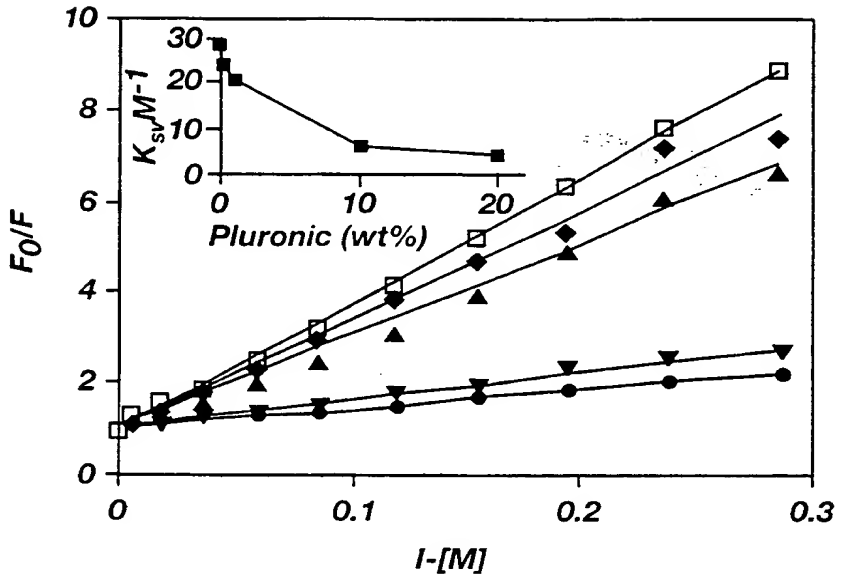
10
8
6
4
2
0
F_0/F
0
0.1
0.2
0.3
I-[M]
30
20
10
0
$K_{sv}M^{-1}$
0
10
20
Pluronic (wt%)

Fig. 3

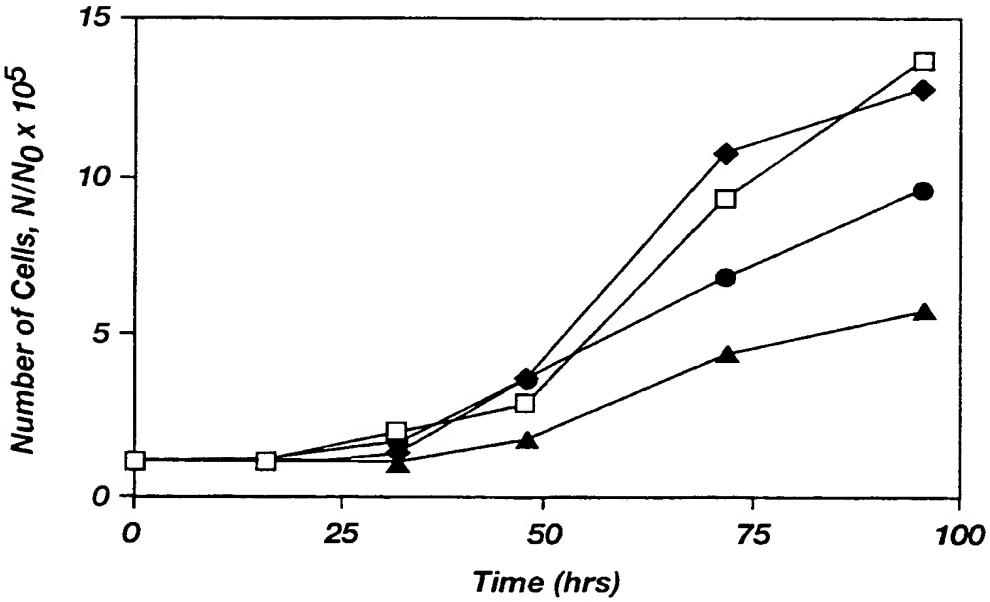
15
10
5
0
Number of Cells, $N/N_0 \times 10^5$
0
25
50
75
100
Time (hrs)

Fig. 4

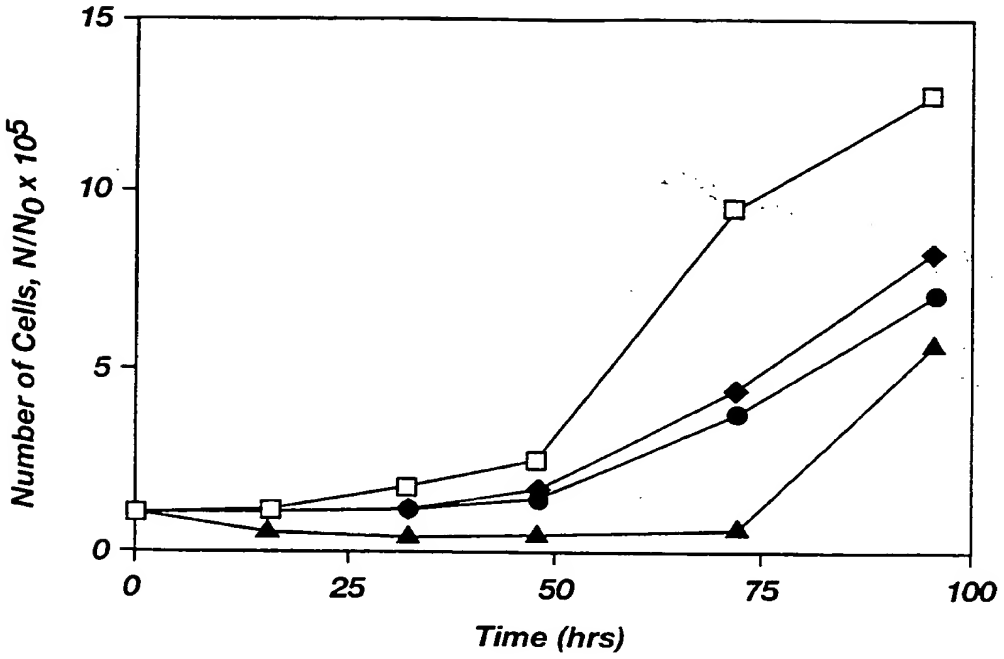
15
10
5
0
Number of Cells, N/N0 x 10^5
0
25
50
75
100
Time (hrs)

Fig. 5

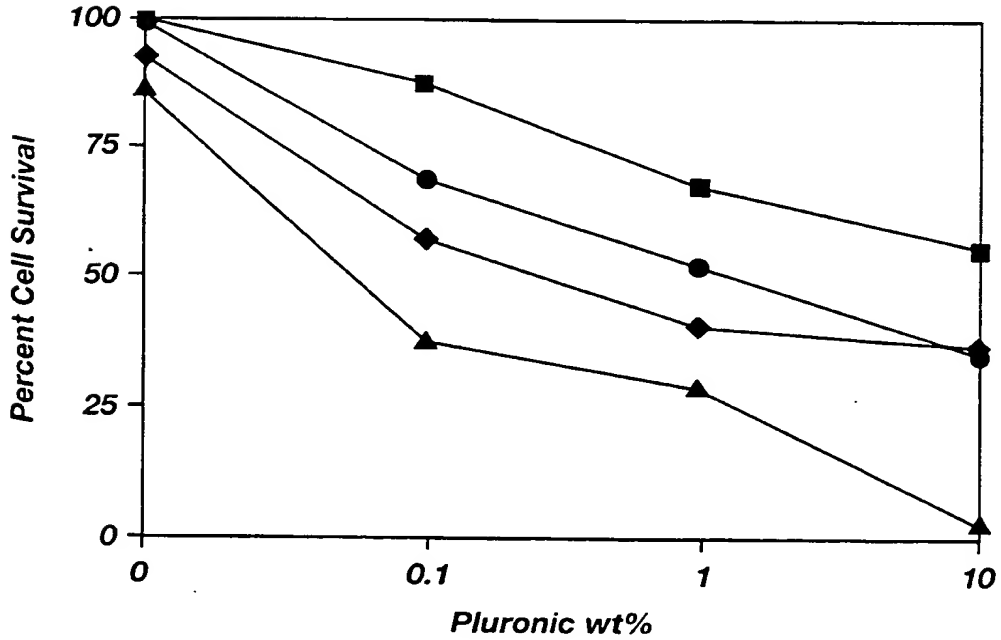
100
75
50
25
0
Percent Cell Survival
0
0.1
1
10
Pluronic wt%

Fig. 6

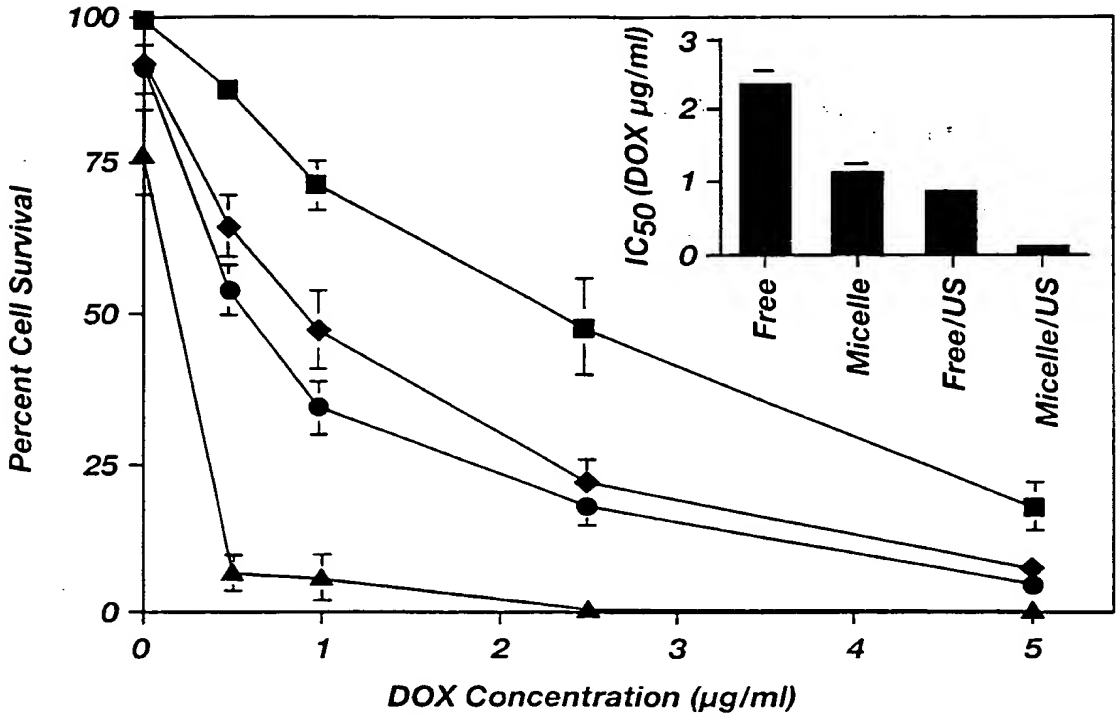
Percent Cell Survival
100
75
50
25
0
0
1
2
3
4
5
DOX Concentration (µg/ml)
IC50 (DOX µg/ml)
3
2
1
0
Free
Micelle
Free/US
Micelle/US

Fig. 7

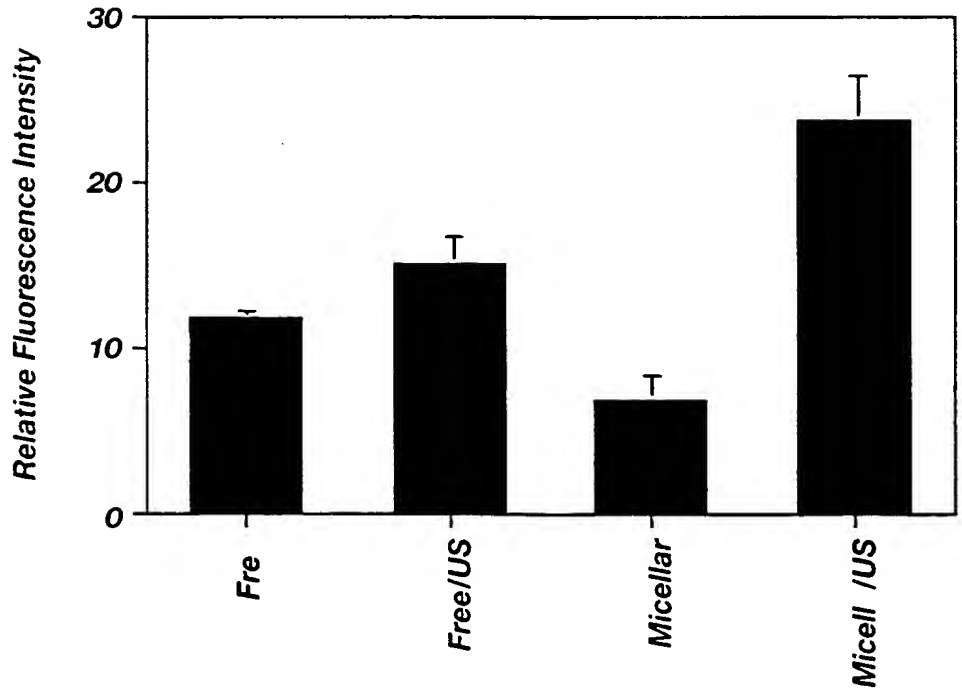
Relative Fluorescence Intensity
30
20
10
0
Fre
Free/US
Micellar
Micell /US

Fig. 8

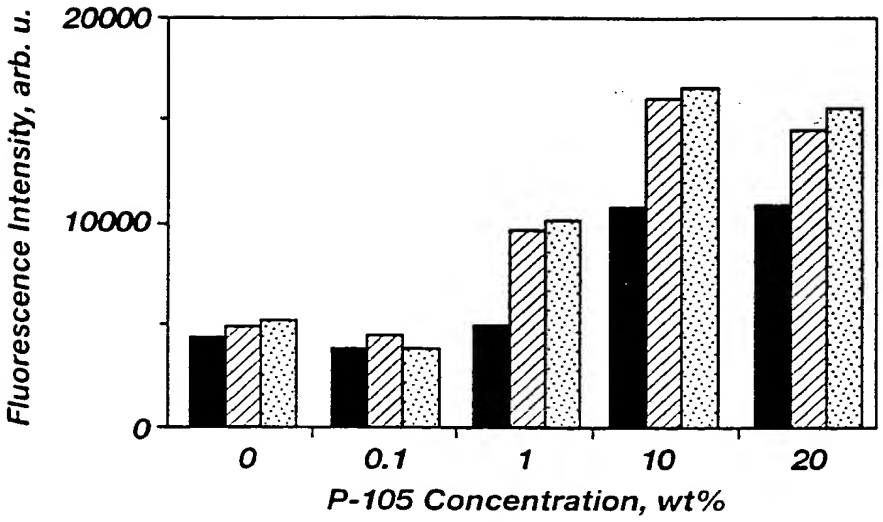
Fluorescence Intensity, arb. u.
20000
10000
0
0
0.1
1
10
20
P-105 Concentration, wt%

Fig. 9

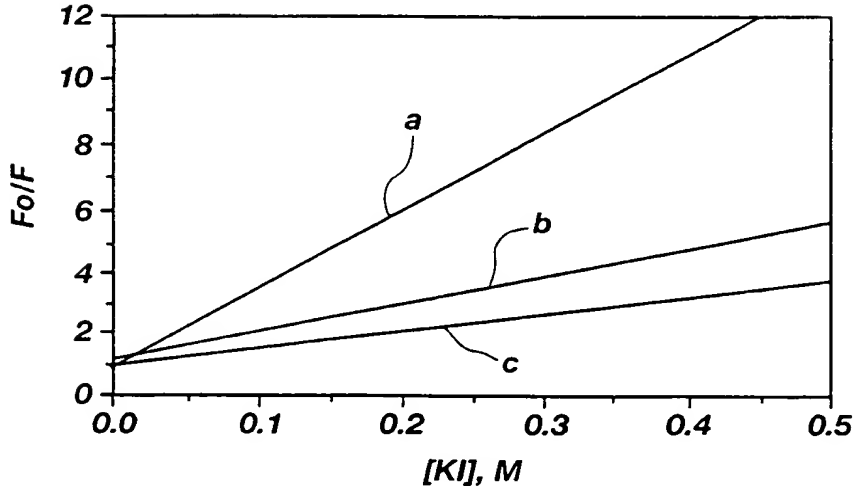
Fo/F
12
10
8
6
4
2
0
a
b
c
0.0
0.1
0.2
0.3
0.4
0.5
[KI], M

Fig. 10

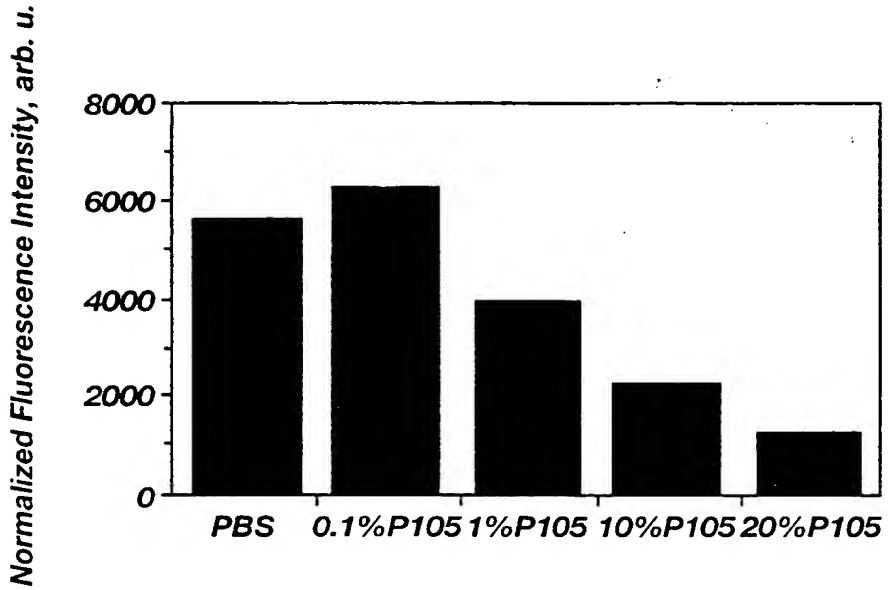
Normalized Fluorescence Intensity, arb. u.
8000
6000
4000
2000
0
PBS
0.1%P105
1%P105
10%P105
20%P105

Fig. 11

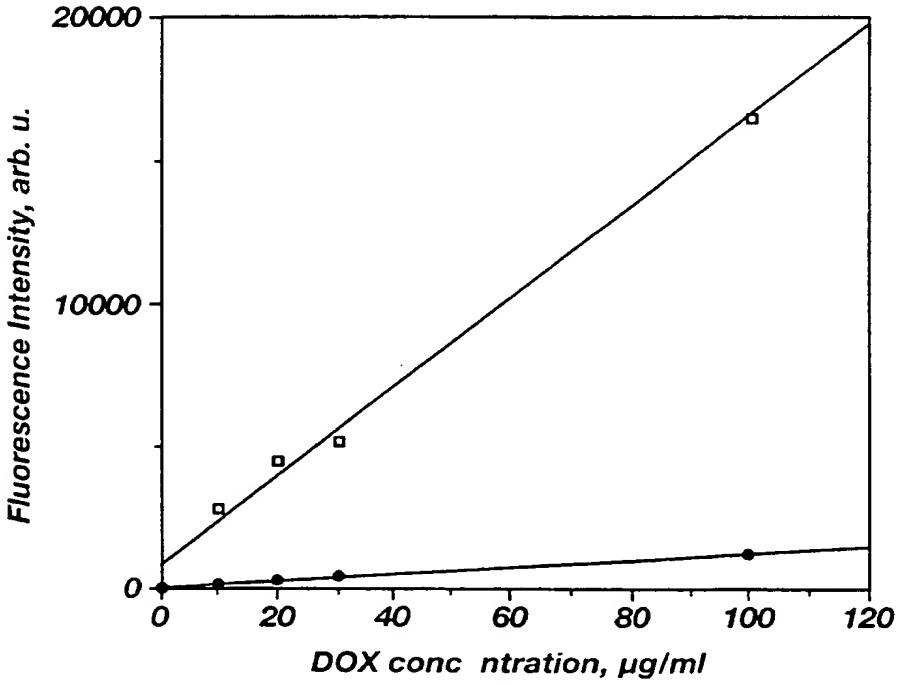
Fluorescence Intensity, arb. u.
20000
10000
0
0
20
40
60
80
100
120
DOX conc ntration, µg/ml

Fig. 12

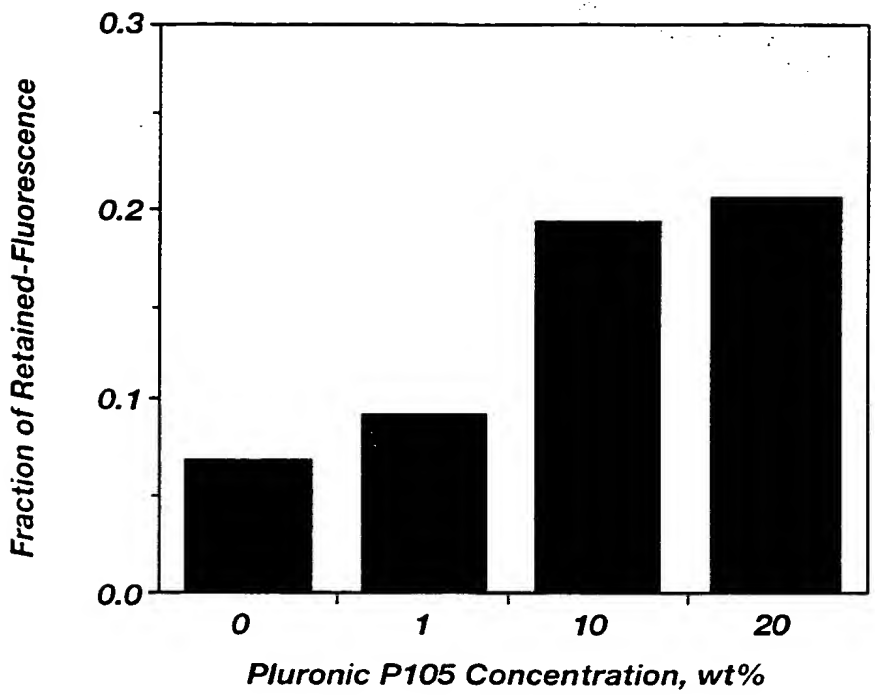
0.3
0.2
0.1
0.0
Fraction of Retained-Fluorescence
0
1
10
20
Pluronic P105 Concentration, wt%

Fig. 13

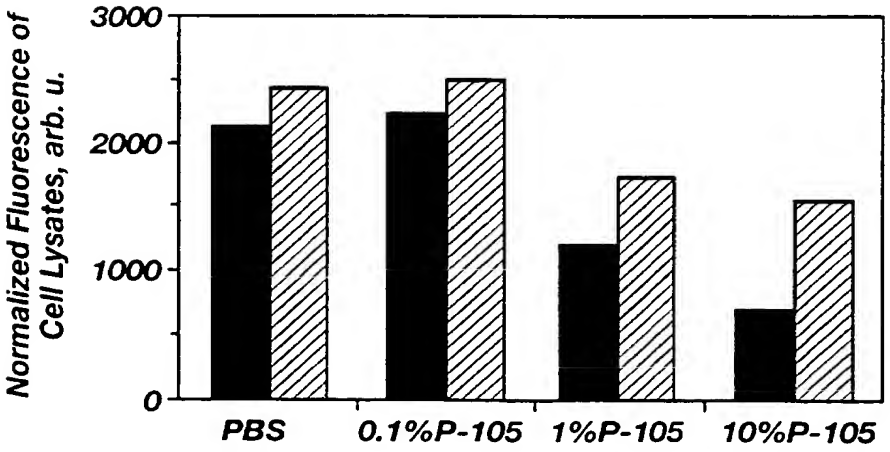
3000
2000
1000
0
Normalized Fluorescence of Cell Lysates, arb. u.
PBS
0.1%P-105
1%P-105
10%P-105

Fig. 14

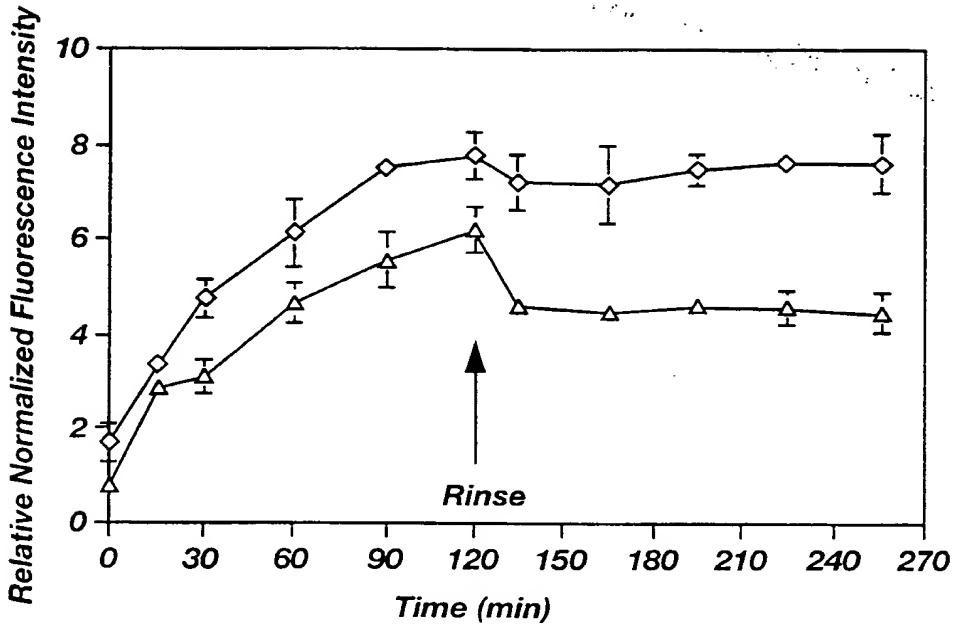
Relative Normalized Fluorescence Intensity
10
8
6
4
2
0
Rinse
0
30
60
90
120
150
180
210
240
270
Time (min)

Fig. 15

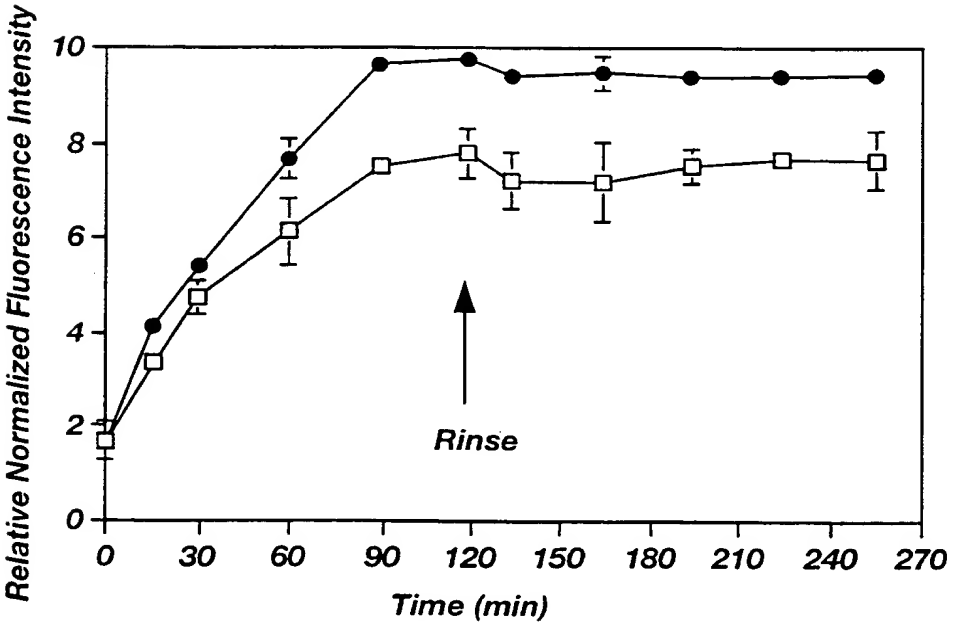
Relative Normalized Fluorescence Intensity
10
8
6
4
2
0
Rinse
0
30
60
90
120
150
180
210
240
270
Time (min)

Fig. 16

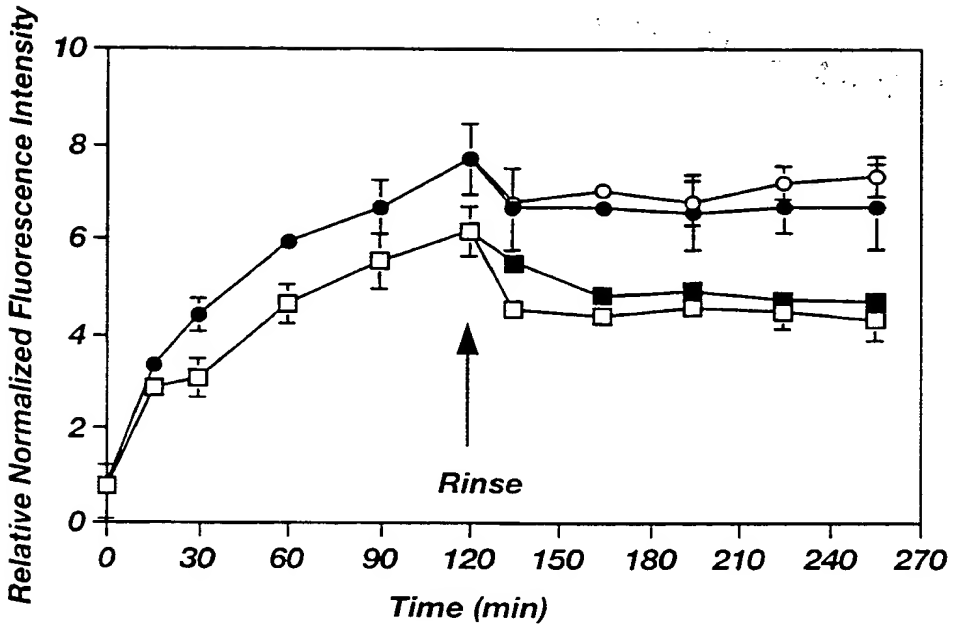
Relative Normalized Fluorescence Intensity
10
8
6
4
2
0
0
30
60
90
120
150
180
210
240
270
Rinse
Time (min)

Fig. 17

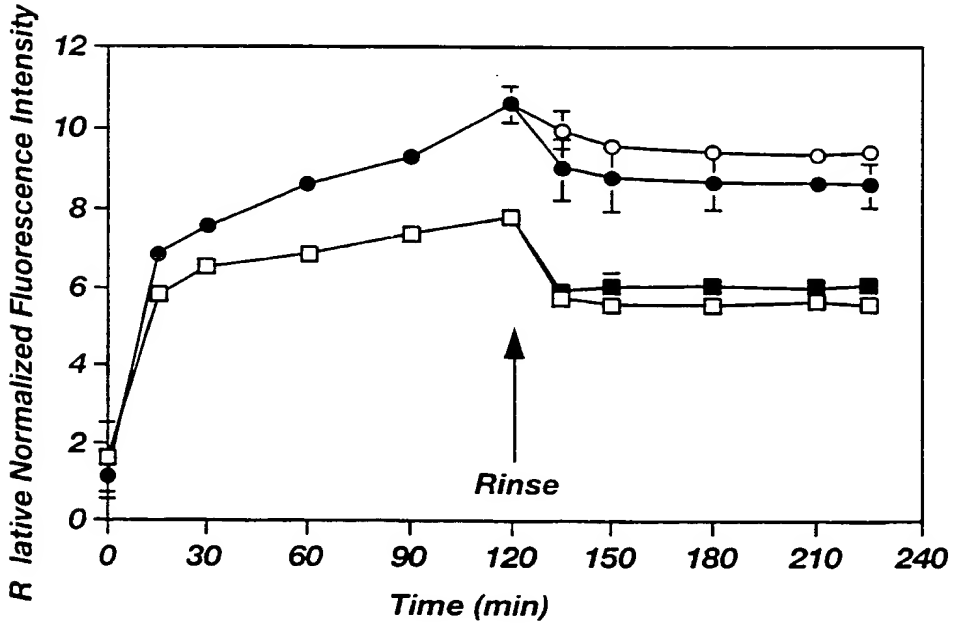
R lative Normalized Fluorescence Intensity
12
10
8
6
4
2
0
0
30
60
90
120
150
180
210
240
Rinse
Time (min)

Fig. 18

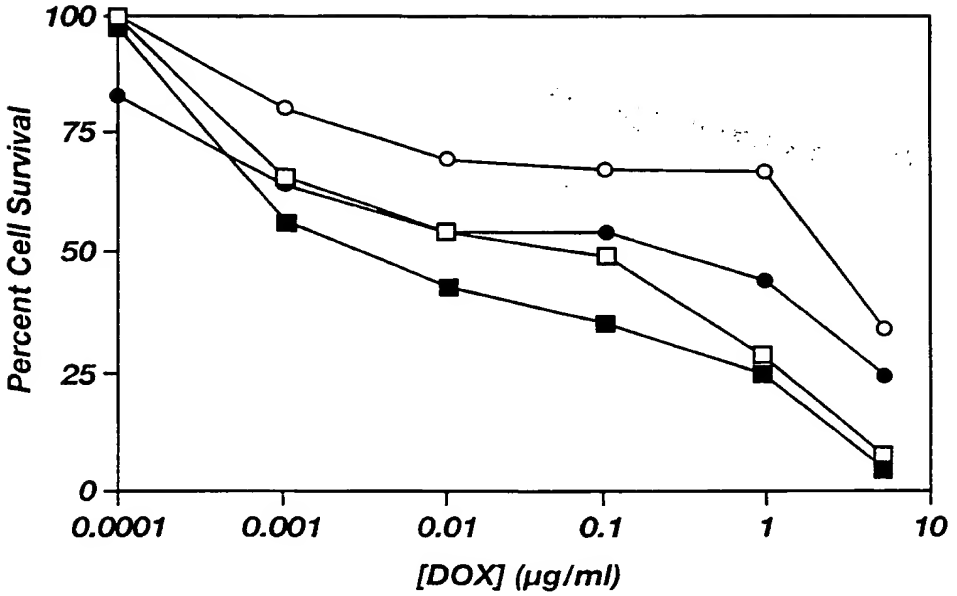
100
75
50
25
0
Percent Cell Survival
0.0001
0.001
0.01
0.1
1
10
[DOX] (µg/ml)

Fig. 19

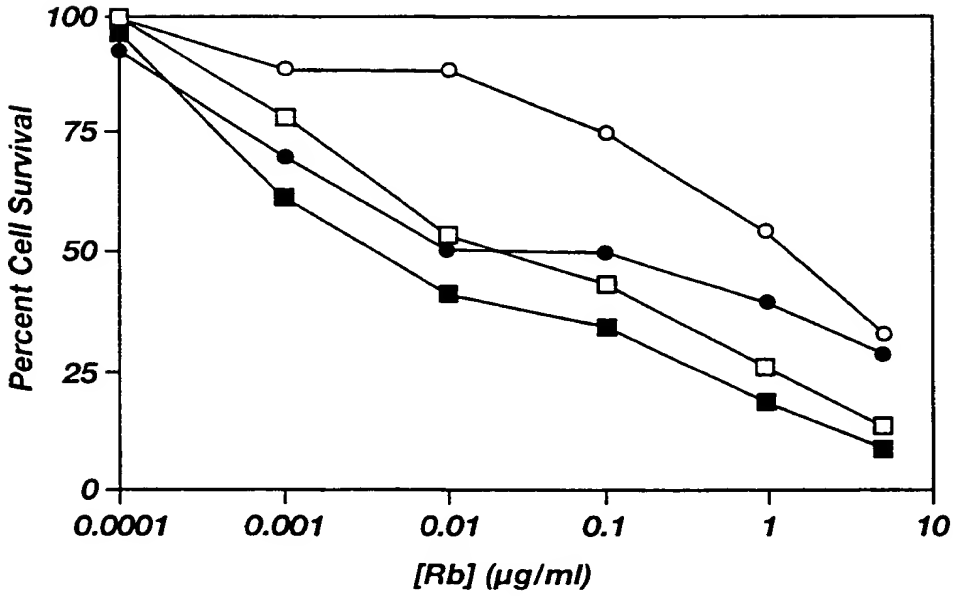
100
75
50
25
0
Percent Cell Survival
0.0001
0.001
0.01
0.1
1
10
[Rb] (µg/ml)

Fig. 20

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING AMENDMENTS OF THE CLAIMS

(PCT Rule 62 and Administrative Instructions, Section 417)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as International Preliminary Examining Authority

Date of mailing (day/month/year)	
01 June 1999 (01.06.99)	
International application No. PCT/US98/20046	International filing date (day/month/year) 23 September 1998 (23.09.98)
Applicant UNIVERSITY OF UTAH RESEARCH FOUNDATION et al	

The International Bureau hereby informs the International Preliminary Examining Authority that no amendments under Article 19 have been received by the International Bureau (Administrative Instructions, Section 417).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Dominique DELMAS

Telephone No. (41-22) 338.83.38

09/509255
5690

RECEIVED

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference T5986.PCT	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US98/20046	International filing date *(day/month/year)* 23 SEPTEMBER 1998	Priority date *(day/month/year)* 23 SEPTEMBER 1997
International Patent Classification (IPC) or national classification and IPC IPC(7): A61K 9/10, 47/32 and US Cl.: 424/400; 514/772.4, 937		
Applicant UNIVERSITY OF UTAH RESEARCH FOUNDATION		

MP
3700

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

   [X] This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 15 sheets.

3. This report contains indications relating to the following items:

   I [X] Basis of the report

   II [ ] Priority

   III [ ] Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV [ ] Lack of unity of invention

   V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI [X] Certain documents cited

   VII [ ] Certain defects in the international application

   VIII [ ] Certain observations on the international application

CORRECTED VERSION

Date of submission of the demand	Date of completion of this report
15 APRIL 1999	27 DECEMBER 1999
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer Edward J. Webman
Facsimile No. (703) 305-3230	Telephone No. (703) 308-1234

Form PCT/IPEA/409 (cover sheet) (January 1994)*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US98/20046

I. Basis f the report

1. This report has been drawn on the basis of *(Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments)*:

☐ the international application as originally filed.

☒ the description, pages (See Attached) , as originally filed.
pages _____ , filed with the demand.
pages _____ , filed with the letter of _____.
pages _____ , filed with the letter of _____.

☒ the claims, Nos. (See Attached) , as originally filed.
Nos. _____ , as amended under Article 19.
Nos. _____ , filed with the demand.
Nos. _____ , filed with the letter of _____.
Nos. _____ , filed with the letter of _____.

☒ the drawings, sheets/~~fig~~ (See Attached) , as originally filed.
sheets/~~fig~~ _____ , filed with the demand.
sheets/~~fig~~ _____ , filed with the letter of _____.
sheets/~~fig~~ _____ , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

☒ the description, pages NONE.

☒ the claims, Nos. NONE.

☒ the drawings, sheets/~~fig~~ NONE.

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US98/20046

V. Reasoned statement under Article 35(2) with regard to nov lty, inventive step r industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	1-8, 13-31	YES
	Claims	9-12	NO
Inventive Step (IS)	Claims	1-8, 13-31	YES
	Claims	9-12	NO
Industrial Applicability (IA)	Claims	1-31	YES
	Claims	NONE	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-8 and 13-31 meet the criteria of PCT Article 33(2) and 33(3) because the prior art neither teaches nor suggests the claimed invention.

Claims 1-31 meet the criteria of PCT Article 33(4) because the claimed invention has industrial utility as a pharmaceutical delivery vehicle.

Claims 9-12 lack novelty under PCT Article 33(2) as being anticipated by MICHAELS.

MICHAELS (abstract) teaches micelles comprising poloxamers to deliver a pharmaceutical agent. Digoxin is disclosed (column 3, line 30).

Applicants aver that MICHAELS does not teach hydrophobic drugs. However, digoxin is almost insoluble in water.

---------- NEW CITATIONS ----------

US 4,182,330 A (MICHAELS) 08 January 1980, see abstract, column 3, line 30.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US98/20046

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

Application No. Patent No.	Publication Date *(day/month/year)*	Filing Date *(day/month/year)*	Priority date (valid claim) *(day/month/year)*
US 5,830,430 A	03 NOVEMBER 1998	21 FEBRUARY 1995	NONE
US 5,698,529 A	16 DECEMBER 1997	06 JUNE 1996	08 OCTOBER 1992

2. Non-written disclosures (Rule 70.9)

Kind of non-written disclosure	Date of non-written disclosure *(day/month/year)*	Date of written disclosure referring to non-written disclosure *(day/month/year)*

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

I. BASIS OF REPORT:

THIS REPORT HAS BEEN DRAWN ON THE BASIS OF THE DESCRIPTION,
PAGES, 1,3-9,13,15,19,21-23, AND 27-29 AS ORIGINALLY FILED.
PAGES, NONE, FILED WITH THE DEMAND.
AND ADDITIONAL AMENDMENTS:
PAGES 2, 10-12, 14, 16-18, 20, 24-26, FILED WITH THE LETTER OF 13 OCTOBER 1999.

THIS REPORT HAS BEEN DRAWN ON THE BASIS OF THE CLAIMS,
NUMBERS, NONE, AS ORIGINALLY FILED.
NUMBERS, NONE, AS AMENDED UNDER ARTICLE 19.
NUMBERS, NONE, FILED WITH THE DEMAND.
AND ADDITIONAL AMENDMENTS:
CLAIMS 1-31, FILED WITH THE LETTER OF 13 OCTOBER 1999.

THIS REPORT HAS BEEN DRAWN ON THE BASIS OF THE DRAWINGS,
SHEETS, 1-10 , AS ORIGINALLY FILED.
SHEETS, NONE, FILED WITH THE DEMAND.
AND ADDITIONAL AMENDMENTS:
NONE

EXPRESS MAIL LABEL NO. EK435394825US

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

Thorpe, North & Western

PCT

To: ALAN J. HOWARTH
THORPE, NORTH & WESTERN, LLP
P.O. BOX 1219
SANDY, UTAH 84091-1219

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Date of Mailing *(day/month/year)* 05 FEB 1999	
Applicant's or agent's file reference T5986.PCT 1015.AWO	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/US98/20046	International filing date *(day/month/year)* 23 SEPTEMBER 1998
Applicant UNIVERSITY OF UTAH RESEARCH FOUNDATION	

1. [X] The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Edward J. Webman Telephone No. (703) 308-1235

Form PCT/ISA/220 (January 1994)*

(See notes on accompanying sheet)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference T5986.PCT	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US98/20046	International filing date *(day/month/year)* 23 SEPTEMBER 1998	(Earliest) Priority Date *(day/month/year)* 23 SEPTEMBER 1997
Applicant UNIVERSITY OF UTAH RESEARCH FOUNDATION		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 2 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (See Box I).

2. [ ] **Unity of invention is lacking** (See Box II).

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] transcribed by this Authority.

4. With regard to the title,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is:

   Figure No. 2
   - [X] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/20046

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 9/10, 47/32
US CL :424/400; 514/772.4, 937
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/400; 514/772.4, 937

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	US 5,795,581 A (SEGALMAN et al) 18 August 1998, col. 4, lines 29-31, col. 6. lines 54-59, col. 10, lines 48-49.	1-25

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 JANUARY 1999	Date of mailing of the international search report 05 FEB 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Edward J. Webman Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*

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PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: ALAN J. HOWARTH
CLAYTON, HOWARTH & CANNON, P.C.
P.O. BOX 1909
SANDY, UTAH 84091-1219

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	04 FEB 2000
Applicant's or agent's file reference T5986.PCT	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US98/20046	23 SEPTEMBER 1998	23 SEPTEMBER 1997

Applicant
UNIVERSITY OF UTAH RESEARCH FOUNDATION

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communicati n to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Articie 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

FEB - 9 2000

Name and mailing address of the IPEA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Edward J. Webman
Facsimile No. (703) 305-3230	Telephone No. (703) 308-1234

Form PCT/IPEA/416 (July 1992)*